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Innate Immunity and Inflammation in Sepsis in a Mouse Model for Binge Drinking

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INNATE IMMUNITY AND INFLAMMATION IN SEPSIS

IN A MOUSE MODEL FOR BINGE DRINKING

By

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Alcohol consumption is a significant risk-factor for mortality in patients with sepsis. This study was carried to investigate the mechanisms by which acute ethanol exposure alters the course of sepsis and the effect of TLR4 signaling.

Ethanol administration decreases resistance to *E. coli* and causes decrease in the ability to clear bacteria both from the peritoneal-cavity as well as the spleen. At early time-points, ethanol also suppresses the production of pro-inflammatory cytokines. TLR4 is dispensable for survival in *E. coli* sepsis but it also contributes to lethality in wild-type mice. Although TLRs have been implicated as an important element of host defense against infections, evidence indicates that these receptors may also play a crucial role in the pathophysiology of sepsis.

DEDICATION

To my parents, *Latief & Naeema*, for being a never ending source of love, strength and support.

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CHAPTER I

INTRODUCTION

Sepsis is a severe, complex and deadly condition which results from an infection of the bloodstream by toxin-producing bacteria. It is also called Systemic inflammatory response syndrome (SIRS) (1). It is a state of disrupted inflammatory homeostasis that is often initiated by bacterial infection (2). It is a major cause of fatalities around the globe, with around 18 million individuals infected annually. In the United States, sepsis is the 10^{th} leading cause of death (3). The mortality rate ranges from 20-60% depending on a number of factors (4, 5). The development and progression of sepsis is multi-factorial, and affects the cardiovascular, immunological and endocrine systems of the body (6). The majority of cases of sepsis are due to bacterial infections, some are due to fungal infections, and very few are due to other causes of infection or agents that may cause SIRS. Common bacterial causes of sepsis are gram-negative bacilli, for example, *E. coli*, *E. corrodens*, *P. aeruginosa*, *S. aureus*, *Streptococcus* species and *Enterococcus* species (7). These infections can originate anywhere in the body and the infecting agents or their toxins then spread directly or indirectly into the bloodstream (8), which helps them to enter into almost any other organ system. SIRS results as the body tries to counteract the damage done by these blood-borne agents (9). The most common areas where the infection originates are the kidneys, the liver, the lungs, the gall bladder, the bowel and

the skin. Sepsis can also be triggered by events such as pneumonia, surgery, burns, and trauma or by conditions such as cancer or AIDS (10). In many cases, the kidneys, liver, lungs, and central nervous system, may stop functioning normally (11). There may also be a decreased blood flow to the kidneys, liver, lungs, and central nervous system (12). Any person can develop sepsis but there are some people who are at a greater risk like those with a weak immune system, those who have wounds or injuries, those with cancer, diabetes and AIDS or elderly patients (13).

The complexity of sepsis makes its clinical study and its therapeutics difficult. The molecules and processes leading to the lethal outcome of sepsis have not yet been fully understood. A significant risk factor for mortality in patients with sepsis is the acute consumption of alcohol (14, 15). Scientists have developed various animal models in an effort to create reproducible systems for studying sepsis pathogenesis and preliminary testing of potential therapeutic agents. Most of these models have shown that pro-inflammatory mediators play an important role in lethality in sepsis (16, 17). However, inhibition of these mediators in clinical trials has not improved the outcome significantly for sepsis patients (18). Efforts to study the lethality in sepsis caused by ethanol in a mouse model (19) is a useful approach to identify mechanisms of lethality in sepsis and may help to explain the ineffectiveness of inhibition of any inflammatory mediators. The study carried out here was designed to understand the differences in inflammatory mechanisms that exist at a time point of 2 hours after ethanol and *E. coli* administration to two different strains of mice, wild type C3H/HeOuJ and the naturally occurring TLR4 mutant C3H/HeJ mice. A few studies at higher time points have already been carried out

in our laboratory which, together with results reported here, has given us some insight into the different mechanisms involved.

The model used in this study is intended to represent sepsis caused by microbial contamination of the peritoneal cavity. A variety of immunological parameters are inhibited and resistance to infection is decreased by chronic and acute exposure to ethanol (19, 20, 21, 22). Acute exposure to ethanol leads to suppression of inflammatory responses in both humans (23, 24, 25) and animal (26, 27, 28) models. Patients with sepsis caused by microbial contamination of the peritoneal cavity following acute ethanol exposure have reported increased rates of infection (22). *E. coli* is one of the most frequently isolated bacteria from patients with sepsis (14). In experimental models, decreased resistance to microbes is evident soon after challenge, suggesting that innate immunity can be adversely affected by ethanol (29) Some studies regarding inhibition of inflammatory responses as a cause of decreased resistance to microbes have been carried out by researchers but they have been only a few in number (19, 30, 31). Studies involving the inhibition of inflammatory responses can reveal the mechanisms by which inflammation promotes bacterial clearance and host survival because the effects of inhibiting a number of those mechanisms simultaneously are revealed by ethanol. In this instance, inhibiting production of several mediators at the same time is beneficial because it will provide an indication of a set of mediators that contribute to lethality. In the present study, evidence is presented indicating mice treated with ethanol exhibit suppression of several mediators and processes of inflammation early which is followed by an overgrowth of bacteria and possibly a lethal systemic inflammatory response.

Alcohol is the most widely abused substance in the United States and numerous studies have revealed that it contributes to a number of adverse effects on the immune system at high dosages (32). Alcohol has widespread effects on the immune system and leaves abusers at increased risk of a variety of infections (33). Amongst the most consistent and profound effects of acute ethanol exposure are decreased production of pro-inflammatory cytokines and chemokines and decreased production of leukocytes at the sites of infection and inflammation (34, 35). Recent studies have demonstrated that recognition of microbial components by TLR expressed by macrophages or other cell types plays a key role in initiating inflammation (36). An increased predisposition to infection among patients with alcohol use problems may also mediate an association with sepsis. Several studies have examined the association between alcohol use disorders and the incidence of severe infections that could reasonably be considered sepsis (37). Multiple human and animal studies have demonstrated abnormal immunity as a result of alcohol exposure (4, 38). This includes abnormalities in innate and adaptive immunity; cellular and humoral responses; the functioning of neutrophils, monocytes, macrophages and lymphocytes; and cytokine and chemokine profiles. Such differences could lead to an increased predilection to infection and once established an increased risk of systemic complications. Alteration of glutathione homeostasis may be an additional mechanism by which alcohol abuse predisposes septic patients to organ failure (39).

Immunity has been usually divided into innate immunity and adaptive immunity. While innate immunity refers to the non-specific immune system and is mediated by the action of macrophages and neutrophils, adaptive immunity is mainly constituted of T and B cells, which are clonally disseminated and known for their specificity and memory.

Since innate immune system lacks specificity, major focus of immune response studies has been on T and B cells and not on innate immunity. However, there has been a recent focus on innate immunity because of the accumulating evidence which has indicated that insects and mammals share many facets of innate immune systems, and it plays a crucial role in the immune response even in mammals (40). It has also been discovered that the innate immune system plays an advisory role in the adaptive immune response in mammals (41).

The innate immune system plays a crucial role in the initial phase of microbial detection. The discovery of toll-like receptors (TLR's) has revolutionized our understanding of how innate immune system recognizes different micro-organisms and how innate immunity gets activated. Toll-like receptors are proteins, which are composed of leucine rich repeats, involved in ligand recognition and a toll/interleukin-I receptor (TIR) domain, involved in signaling that play a critical role in the innate immune system. They activate the innate immune system by identifying specific patterns of microbial components called the pathogen-associated molecular patterns (PAMP's) that activates the production of proinflammatory cytokines and therefore initiate pathogen-specific immune responses (42, 43). Each toll-like receptor has a unique domain that allows specific ligand recognition. So far, thirteen TLRs (TLR1 to TLR13) have been identified in humans and mice together. They also have different adapters to reciprocate to activation and are located both at the cell surface as well as on internal cellular components. Myeloid differentiation primary response gene (MyD88) is essential for most TLR's signaling and involved in nuclear factor-B (NF-kB) and mitogen-activated protein kinase (MAPK) activation and pro-inflammatory gene expression (44). TLR4 is

one of the thirteen toll-like receptors. It is a protein in humans encoded by the *TLR4* gene. It plays a fundamental role in the recognition of lipopolysaccharide (LPS) from gram-negative bacteria. Once the innate immune system is activated by the microbial ligands, immune cells can produce signaling molecules called cytokines which cause inflammation and in the case of bacteria, it might be phagocytosed and digested (45). It is quite interesting that toll-like receptors seem to be involved in the cytokine production and cellular activation only in response to micro-organisms and do not play any major role in their adherence and phagocytosis. Most of the responses activated through TLR4 and other toll-like receptors are inhibited by acute ethanol exposure and it is believed that this contributes to decreased resistance to infection (19, 46, 47, 48). To test this assumption, effects of ethanol in wild-type mice were compared to its effects in mutant-TLR4 mice. The results demonstrate that inhibition of TLR4 signaling by ethanol is probably not a major cause of decreased resistance to sepsis.

C3H/HeJ is a mutant mouse strain that has been found to be hyporesponsive to lipopolysaccharide (LPS) (49). The defective response to LPS in the C3H/HeJ mice is controlled by a single autosomal gene (*Lps*) on mouse chromosome 4 (50, 51). The *Lps* gene has been cloned and shown to encode TLR4. The TLR4 mutation in C3H/HeJ is a mis-sense point mutation which results in the substitution of histidine for a proline that is highly conserved among TLR family members (52, 53, 54). Mice deficient in the TLR4 gene have also been generated by gene-disruption techniques (54). Macrophages and B-cells from TLR4 knock-out mice have been shown to be hyporesponsive to LPS to a similar extent as C3H/HeJ mice, endorsing that TLR4 is required for LPS signaling.

A study carried out by our laboratory earlier has shown that LPS is a major contributor to the induction of cytokines and chemokines by *E. coli* and that the production of cytokines and chemokines is approximately 2 to 15- fold greater in C3H/HeOuJ mice than in the TLR4 mutant C3H/HeJ mice (55). Thus the poor response of C3H/HeJ mice to LPS substantially decreases response of these mice with regard to all tested cytokines and chemokines. This study has also shown that ethanol alone does not induce these cytokines and chemokines to concentrations higher than in control mice.

Cytokines are signaling molecules released by different types of cells and play an important role in innate and adaptive immunity. Cytokines bind to specific cell-surface receptors and produce intracellular signaling that can up- or down-regulate genes, transcription factors, and even other cytokines and cytokine receptors. The effect of a particular cytokine is dependent upon the abundance of the cytokine, the presence of a complementary cell surface receptor, and downstream signals that are activated by receptor binding (56). Overproduction of cytokines can trigger a cytokine storm which is a potentially fatal condition. A distinctive feature of cytokines is that they are usually produced only in response to stimulation (57). Usually their production cycle lasts a few hours to a few days in the normal state, but in an infectious state, it is possible for cytokine production to be prolonged (58). Basically all cells can produce cytokines in response to various stimuli (59). The type of cytokines a cell produces depends entirely on the kind of stimulus, its nature and the presence of other factors like other cytokines, hormones, etc (60). Potency is a key attribute of cytokines (61). They are bioactive at very low concentrations because of the high affinity of their receptors and signaling does not require high receptor occupancy. Another interesting feature of cytokines, known as

pleiotropy, is the ability of a single cytokine to perform different actions on different cells (58, 61). Also, the ability of different cytokines to perform a particular function is another important characteristic of cytokines. This is known as 'redundancy' in cytokine function. The most abundant sources of cytokines are the macrophages, T cells, and mast cells (61). In addition to immune signaling, cytokines also play a role in various other functions like growth, cell division, apoptosis, repair, fibrosis, etc (64).

Cytokines have been established to play a critical role in the initiation of inflammatory responses (65). Inflammatory cytokines can be mainly classified into two groups, acute inflammatory cytokines and chronic inflammatory cytokines (66). The cytokines involved mainly in acute inflammation are IL-1, IL-6, IL-8, IL-11, TNF-α, G-CSF, GM-CSF and other chemokines. The chronic inflammation mediating group of cytokines can be subdivided into cytokines involved in humoral responses such as IL-4, IL-5, IL-6, IL-7, and IL-13, and those responsible for cellular responses such as IL-1, IL-2, IL-3, IL-4, IL-7, IL-9, IL-10, IL-12, interferons, TGF-β, and TNF-α and TNF-β. Inflammation in the acute phase is characterized by increased blood flow and vascular permeability and there is accumulation of leukocytes and cytokines. However, the chronic phase is defined as the process of specific humoral and cellular immune responses to the microbes responsible for the infection or injury. Cytokines function through a complex set of functions that are both synergistic as well as antagonistic interactions and produce negative and positive regulatory effects on different target cells (152).

Acute inflammatory cytokines

Cytokines involved in acute inflammatory reactions include IL-1, IL-6, IL-8, IL-11, TNF-α, G-CSF, GM-CSF and other chemokines. Among these, the primary cytokines responsible for acute inflammation induced in animals by microbial lipopolysaccharide and other mediators of septic-shock are IL-1 and TNF-α (152).

Interleukin-1

The leading producers of interleukin-1 are macrophages, monocytes, fibroblasts, dendritic cells, T cells and B cells. IL-1 cytokines play a very important part in the inflammatory response of the body against infection. IL-1 helps in the production of histamine from mast cells at the inflammatory site, which elicits early vasodilation and increase of vascular permeability (68, 69, 152). Fever can also be triggered by IL-1 cytokines by enhancing the production of prostaglandin E2 by the vascular endothelium of the hypothalamus (67) and can stimulate T cell proliferation. They are also important in the regulation of Hematopoiesis.

Interleukin-6

Interleukin-6 is secreted mainly by mononuclear phagocytes, T cells and fibroblasts (73, 74, 75). It is one of the most important mediators of fever and the acute phase immune response. It also acts as a B cell growth factor and promotes their maturation into antibody-producing plasma cells and also plays a part in T cell activation and differentiation. It has been observed that there is an up-regulation in the production of IL-6 in a variety of chronic inflammatory and autoimmune disorders such as type I

diabetes, thyroiditis, rheumatoid arthritis (76, 77), systemic sclerosis (78), mesangial proliferative glomerulonephritis and psoriasis, cardiac myxoma, renal cell carcinoma, multiple myeloma, lymphoma, and leukemia (76, 152). Its role as an anti-inflammatory cytokine is mediated through its inhibitory effects on IL-1 and TNF-α, and activation of IL-10 (152).

Interleukin-11

Interleukin-11 is mainly produced by bone marrow stromal cells and fibroblasts. Its main role includes increased platelet secretion, induction of acute phase protein secretion, stimulation of T-cell dependent B-cell immunoglobulin production and induction of interleukin-6 expression by CD4+ T cells (80, 152). IL-11 has also got a lot of functional similarities with IL-6 and it can even work as a substitute to IL-6 for the generation of certain plasmacytoma cell lines (79).

Interleukin-8 and other chemokines

Interleukin-8 is mainly produced by endothelial and epithelial cells, mononuclear phagocytes, antigen-activated T cells, and even some neutrophils (81, 83). It is one of the most thoroughly studied chemokine and its main inflammatory effect lies in its chemotactic effects on neutrophils and its ability to generate granulocyte activity. It's primary function is to recruit neutrophils to phagocytose the invading antigen which trigger the toll-like receptors. Interleukin-8 and other chemokines are a part of a chemotactic cytokine family and are in-charge for the chemotactic migration and activation of monocytes, lymphocytes, basophils, eosinophils and neutrophils at the sites

of inflammation (81, 82). Chemokines have been implicated in inflammatory conditions from acute neutrophil-mediated conditions such as acute respiratory distress syndrome to allergic asthma, arthritis, psoriasis, and chronic inflammatory disorders (152). IL-8 can be detected in synovial fluid from patients with various inflammatory rheumatic diseases (84, 85), and mucosal levels of IL-8 are elevated in patients with active ulcerative colitis (86, 152).

Some other chemokines like RANTES, MCP-1, MCP-2, MIP-1, and MIP-2 also play important roles in acute inflammation through their mutual effects on cell migration. MCP-1 is a chemokine which is found in the supernatants of blood mononuclear cells. Its production in monocytes is enhanced by inflammatory cytokines. MIP-1α and MIP-1β induce monocyte and T lymphocyte migration. MIP-1α, MCP-1, and MIP-2 have been implicated in the pathogenesis of rheumatoid arthritis where they are believed to recruit mononuclear cells into the inflamed regions of the synovium (87, 152).

Interleukin-17

Interleukin-17 is mainly produced by T-helper cells. The most important role of IL-17 is its involvement in inducing and mediating proinflammatory responses. IL-17 is commonly associated with allergic responses and its biological activities include stimulation of many other cytokines like IL-6, IL-8 and various other chemokines (89). The functioning of IL-17 is also essential to a subset of CD4+ T-Cells called T helper 17 (T_h17) cells. Latest studies have also shown that one of the molecules that can serve as a mediator of the T cell response to pathogens may be IL-17 (90, 91,152).

Tumor necrosis factor

Tumor necrosis factors-(TNF) α and β are mainly produced by macrophages, mast cells, fibroblasts and some T-cells (70, 71, 72). Both TNF-α and TNF-β bind to common receptors on the surface of target cells and exhibit several common biological activities. TNF- α can induce fever through the stimulation of PGE2 synthesis by the vascular endothelium of the hypothalamus and also by inducing release of IL-1 (67). TNF- α is also responsible for the induction of acute phase reactant protein production by the liver. Large amounts of TNF are released in response to lipopolysaccharide during sepsis with Gram-negative organisms. The systemic release of these cytokines has been shown to be responsible for the fever and hypotension that characterize septic shock (72,152).

Eotaxin

Eotaxins are molecules produced by cytokine-stimulated epithelial and endothelial cells as well as IL-3- stimulated eosinophils. They are specific chemo-attractants for eosinophils. Eotaxin is implicated in allergic responses and inflammatory bowel disease where its mRNA levels are markedly elevated, especially in ulcerative colitis (88,152).

Colony stimulating factors

Colony stimulating factors (CSF's) are produced largely by monocytes, T-cells, fibroblasts and endothelial cells. They are usually named according to the target cell type whose colony formation in soft agar-cultures of bone marrow they induce (92). Two types of CSF's, granulocyte-CSF (G-CSF) and granulocyte macrophage-CSF (GM-CSF)

are involved in acute inflammation. Both these CSF's can stimulate neutrophils, while GM-CSF can also activate effector functions of eosinophils and mononuclear phagocytes (152).

Chronic inflammatory cytokines

Cytokines usually responsible for chronic inflammatory processes can be divided into two groups, those involved in humoral inflammation and those involved in cellular inflammation. The group of cytokines responsible for humoral inflammation includes IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-13 and transforming growth factor-β (TGF-β), and those involved in cellular inflammation include IL-1, IL-2, IL-3, IL-4, IL-7, IL-9, IL-10, IL-12, interferons (IFNs), IFN- inducing factor (IGIF), TGF-β, and TNF-α and TNF-β (152). Chronic inflammation usually develops after acute inflammation and mainly lasts for weeks or months. During chronic inflammation, cytokine interactions take place and as a result, there is transfer of monocytes to the site of inflammation where macrophage activating factors (MAF), such as IFN- , MCP-1, and other molecules then activate the macrophages while other factors, such as GM-CSF and IFN- , retain them at the inflammatory site (93). The macrophages play an important role in the inflammatory processes by chronically multiplying low levels of IL-1 and TNF that result in fever, sleepiness, anorexia and leukocytosis (152).

Humoral inflammatory response cytokines

Interleukin-9

Interleukin-9 is cytokine produced by CD4+ T helper (TH_2) cells and some B-cell lymphomas. It stimulates cell proliferation and prevents apoptosis. It has a regulatory role in the body as it has been shown to inhibit lymphokine production by IFN- -producing CD4+ T cells. It also promotes the growth of CD8+ T cells (94), enhances the production of immunoglobulins by B cells and also plays a role in the proliferation of mast cells (95, 152).

Interleukin-10

The leading producers of Interleukin-10 are the monocytes, CD4+ T cells, activated CD8+ T cells and also some lymphocytes (96). IL-10 plays a key role in many important biological functions. It effects include the down-regulation of TH_1 cytokines, MHC class II antigens, and co-stimulatory molecules on macrophages (97). It also enhances B cell survival, proliferation, and antibody production. It is also known as a cytokine synthesis inhibitory factor (CSIF) because it inhibits IFN- production by activated T cells. Since IL-10 can be produced by TH_2 cells and inhibits TH_1 function by preventing TH_1 cytokine production, IL-10 is considered a T cell cross-regulatory factor and is therefore also known as an "anticytokine" (98). It is also produced by cytotoxic T-cells to inhibit the actions of NK cells during the immune response to viral infection (152).

Interleukin-13

Interleukin-13 is produced mainly by T-helper type-2 (T_H2) cells. IL-13 enhances monocyte and B lymphocyte differentiation and proliferation, increases CD23 expression, and induces IgG4 and IgE class switching (100). It also displays anti-inflammatory activities by inhibiting the production of inflammatory cytokines, such as IL-1β, IL- 8, IL-6 and TNF-α (99). Inhibition of inflammatory cytokine production is also a characteristic of two other cytokines produced by T_H2 lymphocytes, namely IL-4 and IL-10 (152).

Transforming Growth Factor-β

The transforming growth factor- β is a cytokine which is present in three isoforms known as the TGF- β1, β2, and β3. This cytokines family is mainly produced by monocytes, T cells and platelets. TGF-β is usually stored in platelets and is released at the site of injury upon degranulation. It then attracts monocytes and other leukocytes to the injury site and participates in the initial step of chronic inflammation. It also regulates its own production and the expression of integrins resulting in enhanced cell adhesion. TGF-β inhibits T cell and NK cell proliferation and activation and may play an important role in inflammation (101). It inhibits collagenase production and it may also result in unregulated tissue repair if the expression is prolonged. Studies have shown that it may have a role in mesangial proliferative glomerulonephritis, diabetic pulmonary fibrosis, and systemic sclerosis (102, 103, 152).

Cellular inflammatory response cytokines

Interleukin-12

Interleukin-12 is secreted by macrophages, dendritic cells, activated B cells and other antigen-presenting cells (APS's). It was previously also known as natural killer cell stimulatory factor (NKSF) and cytotoxic lymphocyte maturation factor (CLMF). IL-12 plays an important role in the activities of natural killer cells and T lymphocytes. It helps in the enhancement of the cytotoxic activity of NK cells and CD8+ cytotoxic T lymphocytes, in the induction of IFN- production by NK cells and T cells, and inhibition of IgE synthesis by IL-4-stimulated lymphocytes (104, 105, 106). But its production is also inhibited by IL-4 and IL-10 cytokines. The stimulatory effect of IL-12 on T_H1 development is countered by IL-4, a cytokine which promotes T_H2 cell development. Therefore, IL-12 plays a key role in cell-mediated inflammation and also contributes to the regulation of immunoglobulin production (152).

Interleukin-15

Interleukin-15 is produced by many types of cells including the monocytes and T lymphocytes. It was originally discovered as a molecule having a T cell stimulatory activity (107). It regulates T lymphocyte and NK cell activation and proliferation, as well as CTL and LAK activity (95). It plays a role in the enhancement of B cell proliferation and immunoglobulin production (108). It also acts as a T lymphocyte chemo-attractant. Various studies have also shown that IL-15 may have a role in the induction and

activation of T lymphocytes in the synovium of patients with rheumatoid arthritis where its levels have been found to be elevated (109, 152).

Interferons

Interferons are a group of cytokines which are produced mainly by lymphocytes. They play an important role in a variety of functions like the activation of immune cells, such as natural killer cells and macrophages, increasing the recognition of infection or tumor cells by the up-regulation of antigen presentation to T lymphocytes and increasing the ability of uninfected host cells to resist new infection by microbes (110). The type I interferons include IFN-α and IFN-β. These interferons possess anti-escalative and anti-viral properties and up-regulate MHC class I expression. IFN- is another type of interferon and is known to stimulate MHC class I and II expression and also stimulates various effector functions of mononuclear phagocytes. IFN-α and IFN-β have a common receptor while IFN- has a distinct and specific cell surface receptor. Studies have shown that IFN- plays a role in the pathogenesis of various autoimmune and chronic inflammatory conditions (111) like Type I diabetes mellitus (113, 114), adjuvant-induced arthritis (115), and experimental cerebral malaria (116). In experiments with IFN- knock-out mice, it has been observed that one of primary functions of IFN- *in vivo* appears to be the activation of macrophages to kill intracellular pathogens such as mycobacteria (117, 152).

CHAPTER II

MATERIALS AND METHODS

Mice

Two different strains of mice, C3H/HeJ and C3H/HeOuJ were used. C3H/HeJ mice have a mutant TLR4 gene which yields a protein that is non-responsive to bacterial lipopolysaccharide, which is the naturally occurring ligand for TLR4. The C3H/HeOuJ mice are the wild-type strain which matches the C3H/HeJ strain at every locus other than TLR4. All the mice were obtained from Jackson Labs (Bar Harbor, Maine). They were allowed to acclimatize and to recover from the shipping stress for at least 2 weeks before use in experiments at 8-12 weeks of age. Female mice were used because males fight when group housed and this causes stress, which can affect the results. All the mice were housed in filter-top shoebox cages with 5 mice per cage in a temperature (70-78□ F) and humidity (40-60%) controlled environment. Because of the decreased resistance to some microbes, food, water and bedding for the mice were autoclaved before use for all the mice. Sentinel mice housed in the same room as the mice used in this study were negative for all common infectious agents during the period of this study. The laboratory animal facility and animal research program at Mississippi State University are accredited by the American Association for Accreditation of Laboratory Animal Care. Mice were housed

and used in accordance with the National Institute of Health and Mississippi State University regulations.

Administration of ethanol

Ethanol was administered as a 32% v/v (volume/volume) solution in tissue culture-grade water by oral gavage using an 18-guage stainless steel gavage needle. All the mice were treated with a dosage of 6g/kg ethanol. This dose yields a blood ethanol concentration of ~0.4% which is similar to the blood ethanol levels that occur in ethanol dependent humans **(120)**. Although this blood concentration represents the high-end of the range typically observed in ethanol-dependent persons or binge drinkers, concentrations in this range are not rare. In the naive mice, water alone was used by gavage to control for handling and dosing related stress.

Administration of *E. coli*

Viable *E. coli*, log phase, grown in LB broth was administered intraperitoneally. The dosage of *E. coli* was 2×10^8 per mouse, which is similar to the dosages used by other investigators.

The *E. coli* strain used in this study was isolated from the colon of one of the mice in our specific pathogen free colony. It was characterized by the College of Veterinary Medicine Clinical Microbiology Laboratory as a non-pathogenic *E. coli*. As expected for non-pathogenic bacteria, mice can clear a large number without mortality. However, 2×10^8 per mouse routinely yields 10-20% mortality, indicating that this is a sufficient dosage to identify decreased resistance to sepsis, which would cause higher mortality.

Bacteria for the experiment were prepared starting with a frozen vial, which was one of a set frozen at the same time from the same culture. Bacteria were in log growth phase, which was indicated by a spectrophotometer OD at 650nm and dosages were also estimated using same OD measurements and a standard growth curve. This number was verified by serial dilutions and plate counts and values were always within 10% of the nominal count. This model is expected to be representative of sepsis in humans that begins with the loss of gastrointestinal barrier function which is usually caused by trauma, appendicitis, diminished liver function etc. In human peritonitis, a single species of bacteria often predominates and in approximately half of the cases, *E. coli* is the species isolated in blood cultures (121, 122). Thus, administration of a single strain of indigenous *E. coli* in our model allows more controlled conditions than cecal ligation and puncture but yields peritonitis and sepsis similar to that observed in humans.

Experimental Design

Experiment was designed with a group size of 5 mice. Both the strains of mice were divided into 3 groups each and were grouped on the basis of treatment administered. The first group of mice in each strain remained untreated and was referred to as the naive group. This group served to confirm that the anticipated inflammatory changes were induced by *E. coli*. The second group in each strain was treated with *E. coli* only and the third group was treated with both *E. coli* and ethanol.

Mice were treated by gavage with ethanol or water and immediately challenged with *E. coli* (2×10^8 per mouse). Different groups of mice were anesthetized by inhalation of halothane at 2 hours after *E. coli* challenge.

Sampling of blood, peritoneal lavage and extraction of spleen

Blood samples were obtained retro-orbitally while the animal was under halothane anesthesia. Samples were collected and the serum was removed after centrifugation. Serum was refrigerated until needed for analysis. After euthanasia by continued inhalation of halothane, peritoneal lavage was performed by injection of 1ml of PBS. The abdominal area was massaged to distribute the fluid and to mix the contents of the peritoneal cavity. The skin was dissected away to reveal the peritoneum and a sample from the peritoneal cavity was withdrawn using a syringe with a 25-guage needle. Samples of this fluid were used to quantify bacteria by making serial dilutions in LB agar, kept at 45°C to prevent solidification and plating and performing the plate counts manually. The rest of the peritoneal fluid sample was centrifuged to remove cells and debris and the supernatants were used for differential cell counts using a Coulter Z1 particle counter, for cytospin preparations followed by Wright Giemsa staining and differential cell counts at 600x magnification under an electronic microscope. The rest of the fluid in all of the samples was refrigerated at -20° C until needed for cytokine and chemokine assay. Spleen from all the mice was also collected and weighed and then divided into three almost-equal parts and stored in different vials at -80° C to quantify bacteria by serial dilutions in LB agar kept at the same conditions as the intra-peritoneal fluid, and the other two parts were also stored at -80°C for proteomics and RNA analysis respectively.

Quantifying bacteria

After anesthesia, peritoneal lavage was performed on all the mice. Samples of this fluid were used to quantify the bacteria by making serial dilutions in LB agar, which was prepared fresh and held at 45°C to prevent its solidification. Plating was done and plate counting was performed.

Cytospin Counts

Peritoneal lavage fluid samples were also used for cytospin preparations by using Wright Giemsa staining and differential cell counts at 600x magnification with an oil immersion lens. Cells with three or more bacteria associated were referred to as cells with *E. coli* and cells with less than three *E. coli* were referred to as cells without *E. coli*. This was designed to account for the possibility that some of the bacteria that appeared to be intracellular might actually be on the cell surface.

Cell Counts

The number of nucleated cells in peritoneal fluid as well as spleen was determined by using samples of peritoneal lavage fluid with cells suspended and samples of spleen. Manual lysing reagent was added to lyse the cytoplasmic membrane, leaving only nuclei to be counted. Counts were determined using a Coulter Z1 particle counter.

Cytokine assays

Cytokine and chemokine concentrations in peritoneal lavage fluid were quantified using kits from Millipore Corporation, called MILLIPLEX MAP Mouse

Cytokine/Chemokine - Premixed 32 Plex, 96-Well Plate Assay using standards for each cytokine and chemokine.

Flow cytometry analysis

Spleen samples were used for carrying out flow cytometry using a BD Biosciences FACS Calibur flow cytometer which is a multicolor benchtop flow cytometry system that is capable of both analyzing and sorting.

The different markers used include the CD4, the CD8, the NK1.1 and the cells that express MHC II. The cells labeled by these markers are T helper lymphocytes (CD4), cytotoxic T lymphocytes (CD8), NK cells (NK1.1), B lymphocytes (MHC II) and macrophages (MHC II).

Statistical analysis

All the data analysis was performed using Prism 5.0 software (GraphPad, San Diego, CA). Data with continuous variables were analyzed by analysis of variance (ANOVA), followed by Newman-Keuls post-hoc test to identify differences in group means. Means with a P value of .05 or less were considered significantly different.

CHAPTER III

RESULTS

Acute administration of ethanol suppressed the immune response in both the wild type (C3H/HeOuJ) and TLR4 mutant (C3H/HeJ) mice. The results in Figure 1 indicate the number of viable *E. coli* isolated at 2 hours after challenge from the peritoneal cavity of control and mutant mice. The results demonstrate that ethanol suppressed the clearance of bacteria from the peritoneal cavity of both the strains of mice. Similarly, the results in Figure 2 indicate the number of viable *E. coli* isolated from the spleen at 2 hours after challenge from the peritoneal cavity of both the wild type and mutant mice. The results here show a similar pattern as the peritoneal fluid; however, the numbers are much less than in the peritoneal fluid. Both these results reflect the suppression of the clearance of bacteria by ethanol. It should be noted that 2 hours is very early in the process of infection, and it has been reported previously that the differences in bacterial numbers get larger over time in ethanol treated and control mice (55). In the peritoneal fluid there was a slight increase in bacterial counts in TLR4 mutant mice as compared to control mice. In contrast, a smaller number of bacteria were noted in the spleen of mutant ethanol treated mice than in wild type ethanol treated mice.

Figure 3 shows the cell counts of the peritoneal fluid samples from both the strains. The results in Figure 4 indicate the cell counts of the spleen samples. There was no significant effect of ethanol on cell number in either mouse strain, and there was no significant difference between wild type and TLR4 mutant strains.

The results in Figure 5 & 6 indicate the type of cells in the peritoneal cavity of the mice. As the figures show, most of the cells in the peritoneal cavity were macrophages and there were a small percentage of lymphocytes present in both the control as well as mutant mice. Neither *E. coli* nor ethanol had a significant effect on the percentages of these cells. There was also no significant difference between strains.

The effects of ethanol on cytokine and chemokine production at a time point of 2 hours after *E. coli* challenge are indicated in Figures 7 (a)-(y). The production of all of the cytokines and chemokines, except IL-10 and MIP-2, was significantly decreased by ethanol treatment just before the *E. coli* challenge in wild type mice. In contrast, several cytokines and chemokines (IL-1β, MIP-2, IL-10, IL-17, LIF, RANTES, MIP-1α, TNF-α, Eotaxin, and GM-CSF) were not significantly altered by ethanol in TLR4 mutant mice. This provides the first evidence that most cytokines and chemokines induced through receptors other than TLR4 are not significantly decreased by ethanol. The TLR4 mutation in C3H/HeJ mice significantly decreased production of most of the 25 cytokines and chemokines tested. Only MIG, M-CSF, G-CSF, MCP-1, IP-10, IL-6, IFN- , and Eotaxin were not significantly different in mutant as compared to wild type mice.

Ethanol caused a significant increase in cytokine or chemokine production only in the case of IL-10 in wild type mice and IL-17 in the TLR4 mutant strain.

Figures 8 (a)-(d) represent the flow cytometry data obtained by carrying out flow cytometry analysis of the spleen samples from both the wild type and mutant strains of the mice. Figure 8a shows the CD4 cell percentages, Figure 8b represents the CD8 cell percentages, Figure 8c shows the NK1.1 cell percentages and Figure 8d shows the percentage of cells that express MHC II. The cells labeled by these markers are T helper lymphocytes (CD4), cytotoxic T lymphocytes (CD8), NK cells (NK1.1), B lymphocytes (MHC II) and macrophages (MHC II). Ethanol did not significantly affect the percentage of any of these cells types. However, the TLR4 mutant mice had a modestly higher percentage of CD4, CD8, and MHC II positive cells than wild type mice.

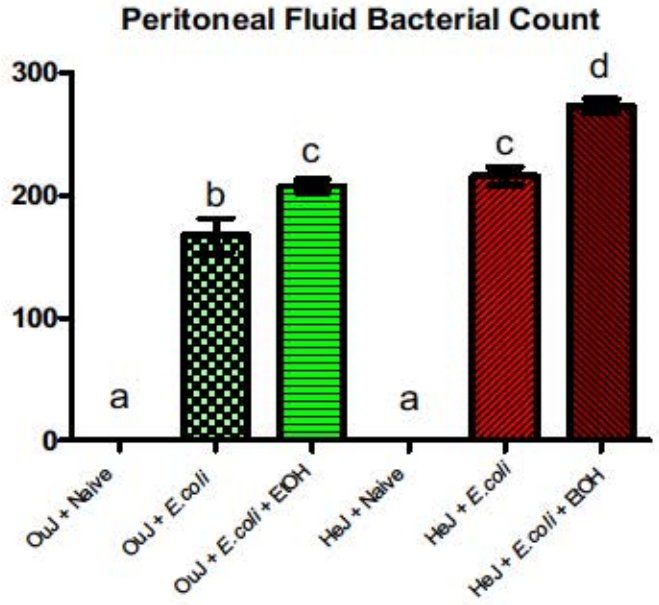


Figure 1 Number of viable *E. coli* isolated at 2 h after challenge from the peritoneal cavity of control (C3H/HeOuJ) and mutant (C3H/HeJ) mice

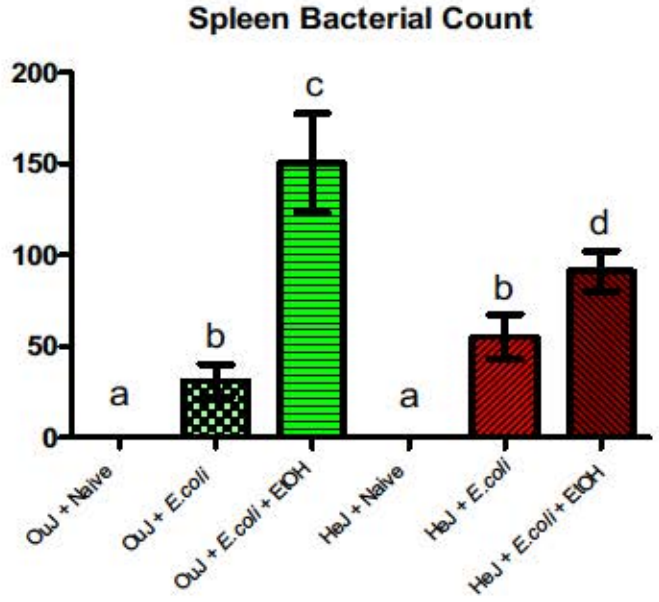


Figure 2 Number of viable *E. coli* isolated at 2 h after challenge from the Spleen of control (C3H/HeOuJ) and mutant (C3H/HeJ) mice

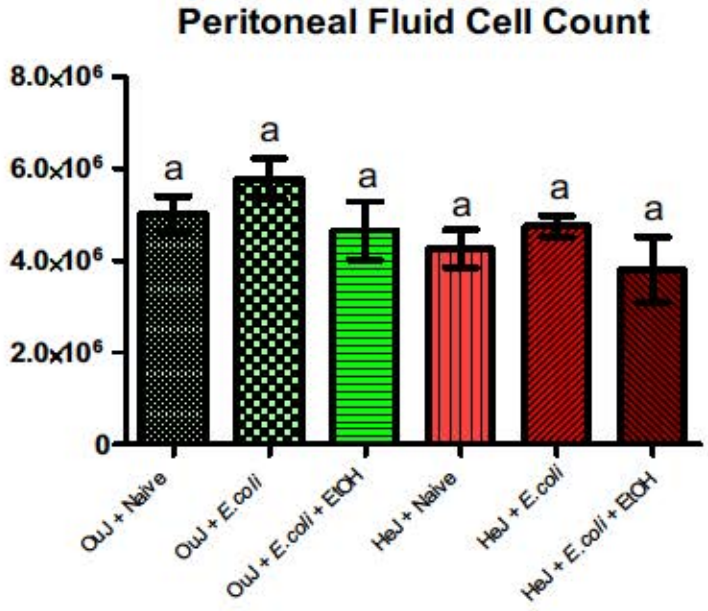


Figure 3 Cell Counts at 2 h after challenge from the peritoneal cavity of control (C3H/HeOuJ) and mutant (C3H/HeJ) mice

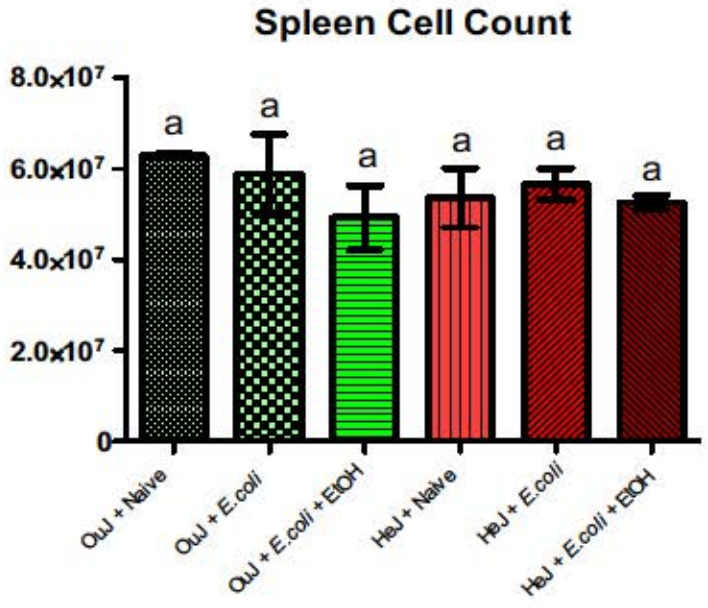


Figure 4 Cell Counts at 2 h after challenge from the Spleen of control (C3H/HeOuJ) and mutant (C3H/HeJ) mice

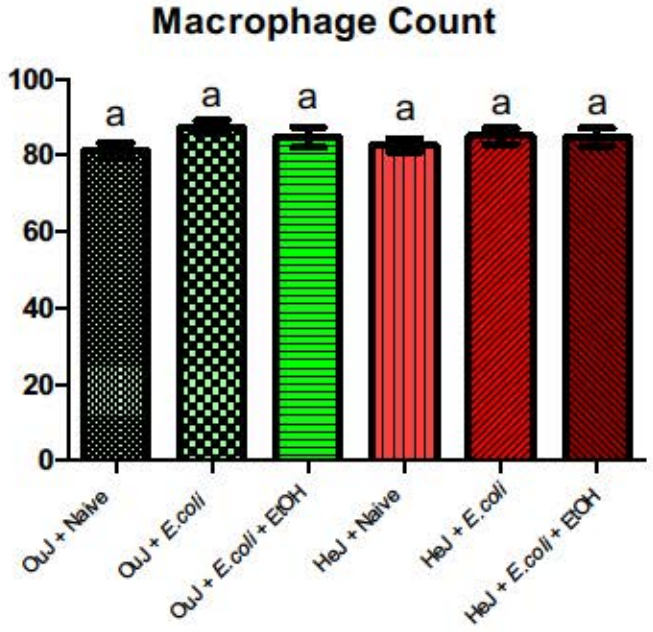


Figure 5 Indicates the percentage of macrophages in the peritoneal cavity of the mice 2 h after *E. coli* challenge

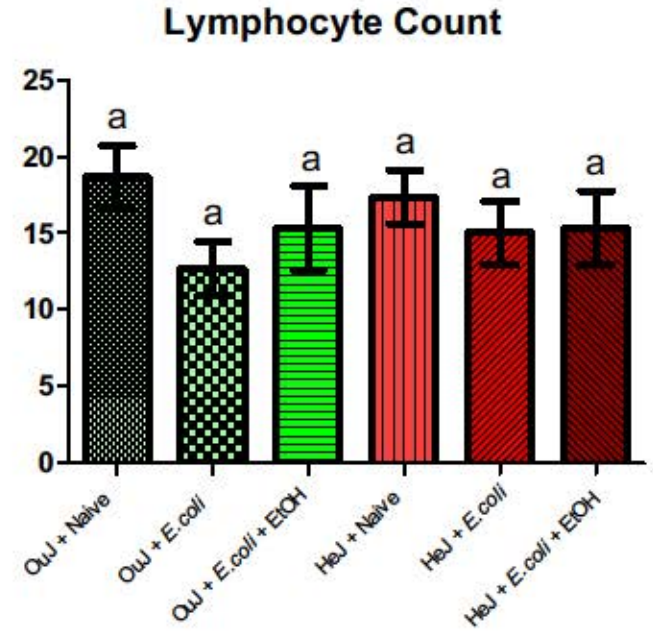


Figure 6 Indicates the percentage of Lymphocytes in the peritoneal cavity of the mice 2 h after *E. coli* challenge

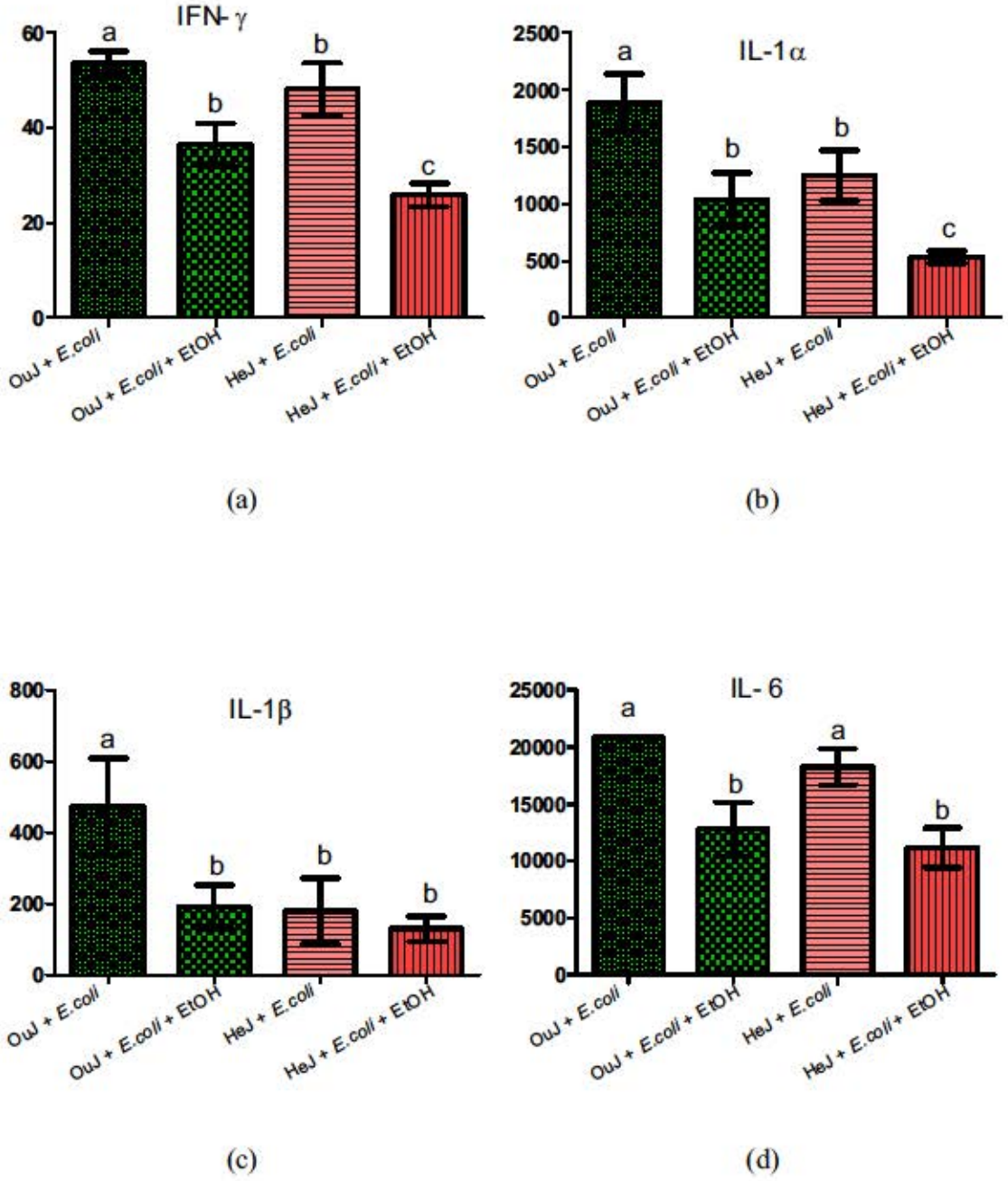
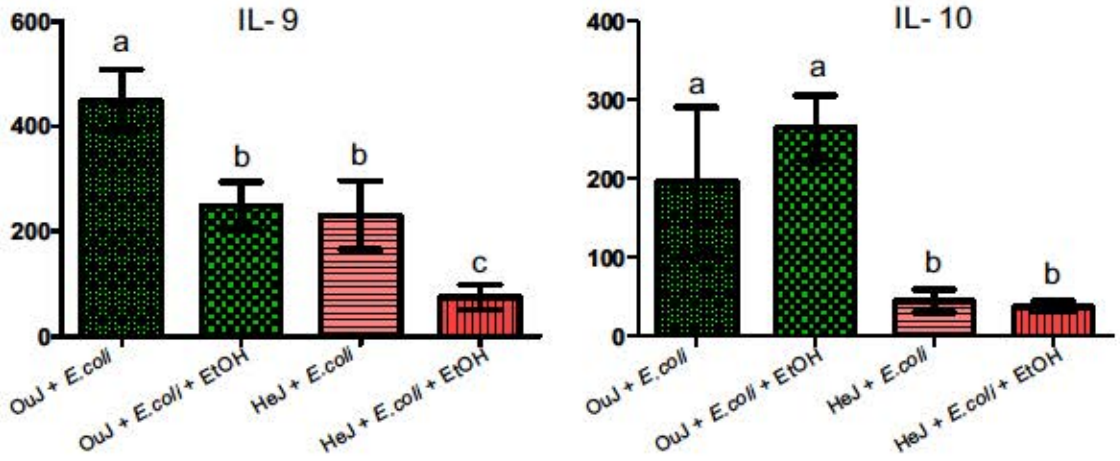


Figure 7 (a)-(y) Indicate the effects of ethanol on cytokine and chemokine production at a time point of 2 h after *E. coli* challenge



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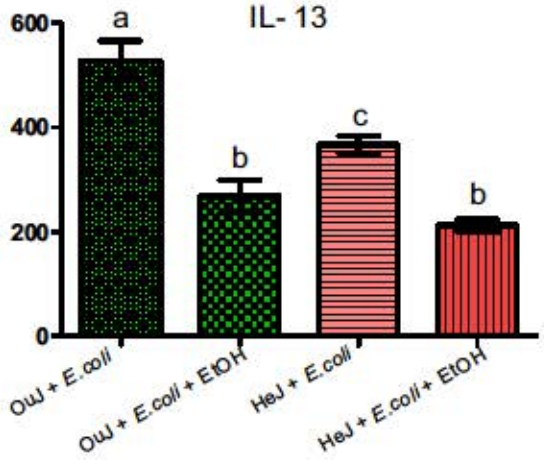

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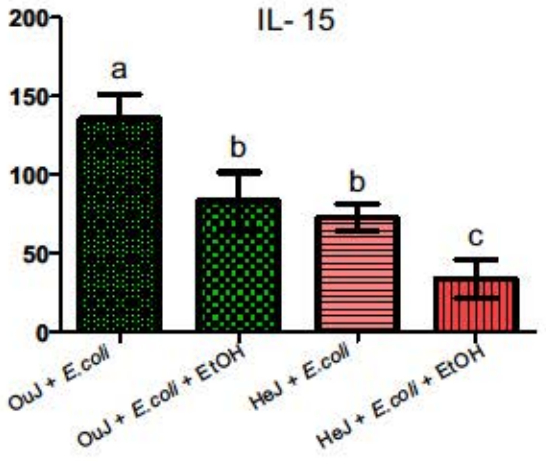

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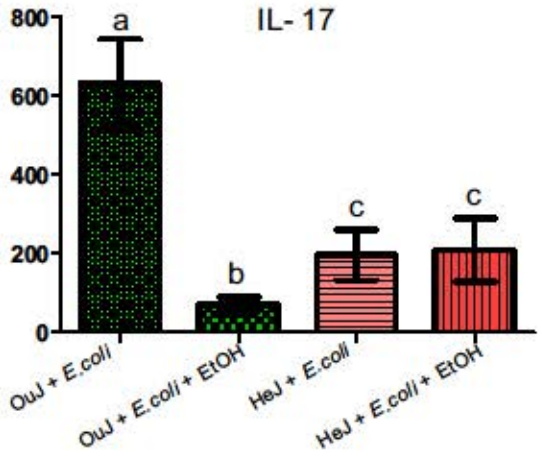

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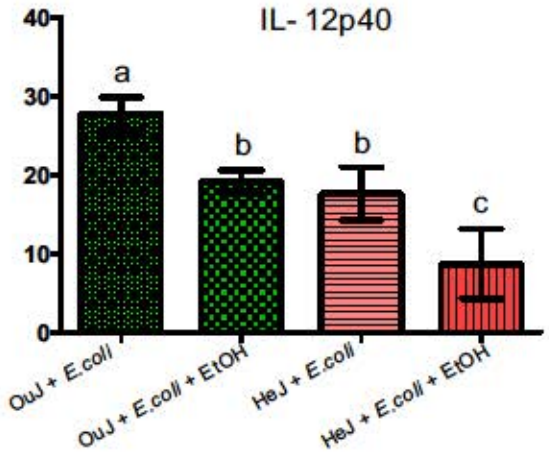

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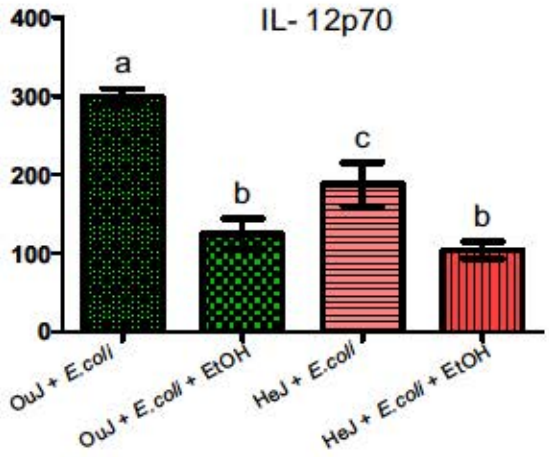

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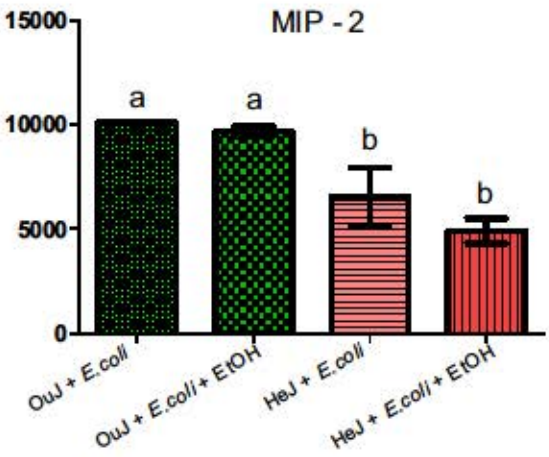

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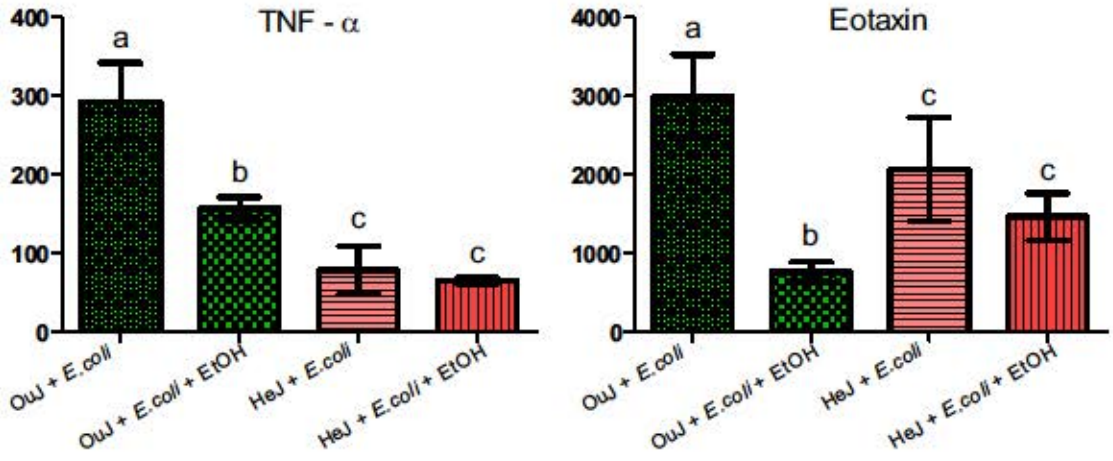

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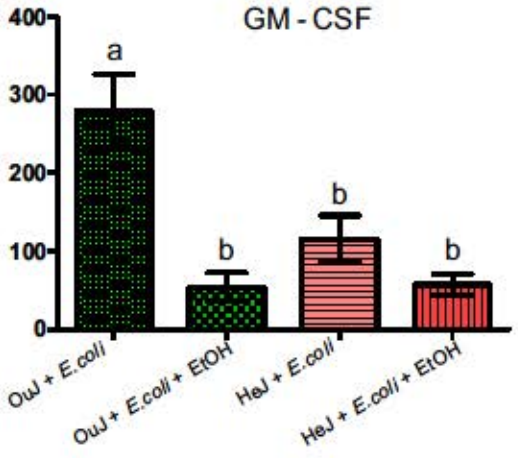

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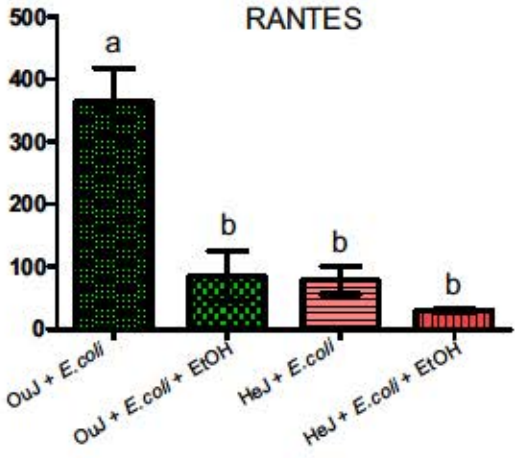

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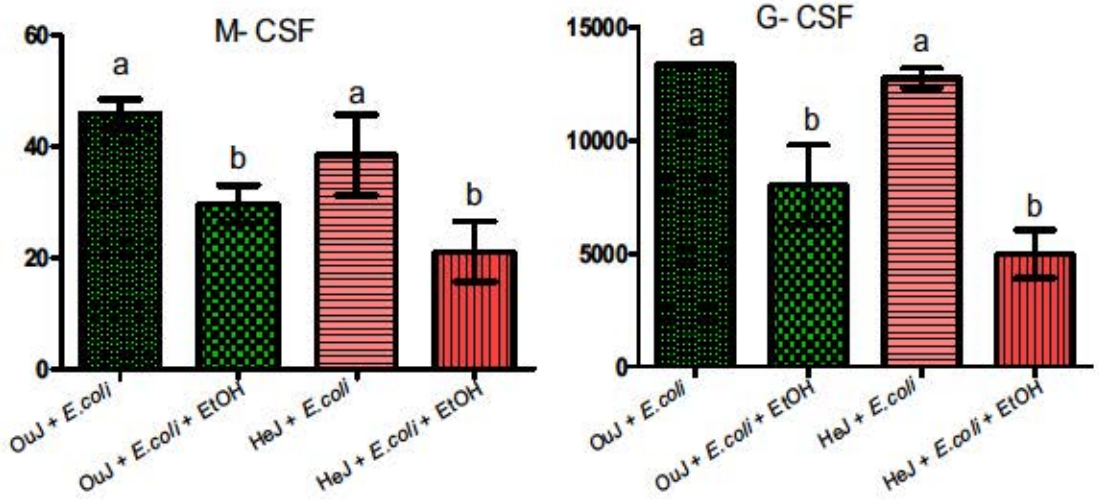

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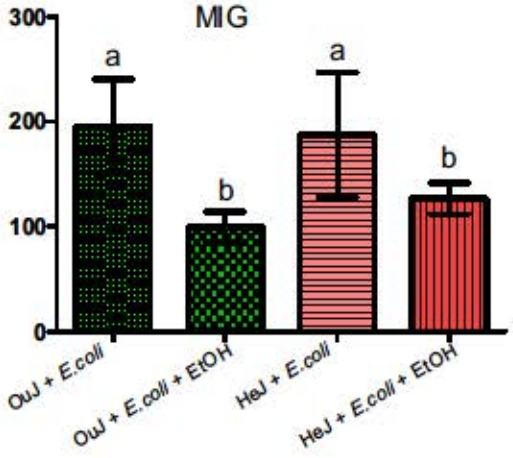

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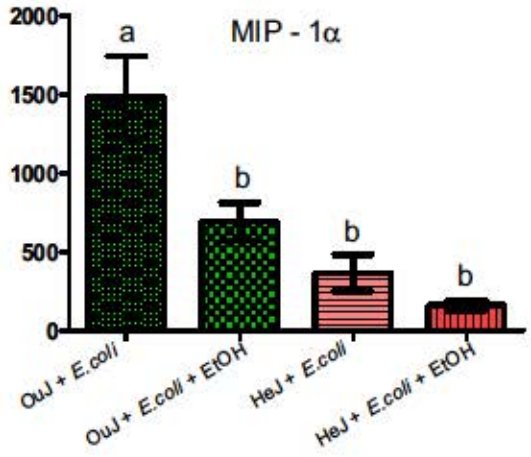

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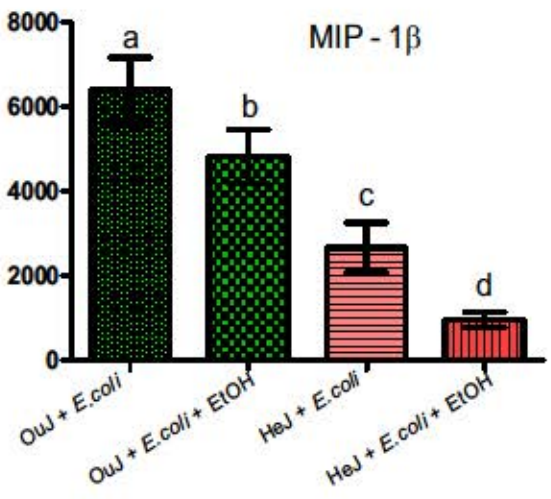

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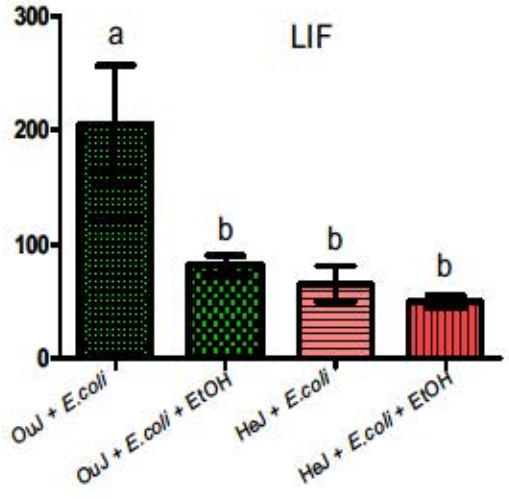

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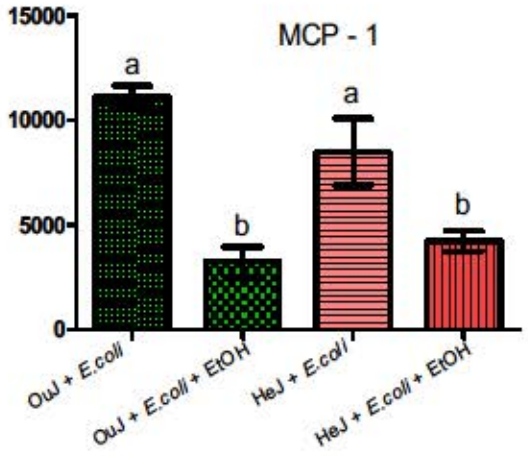

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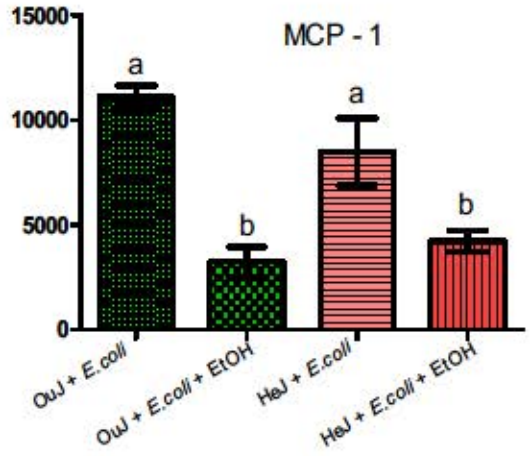

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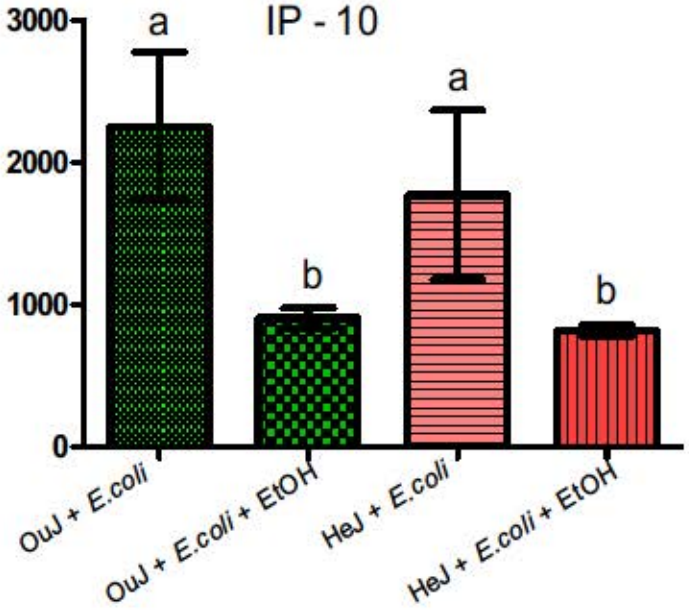

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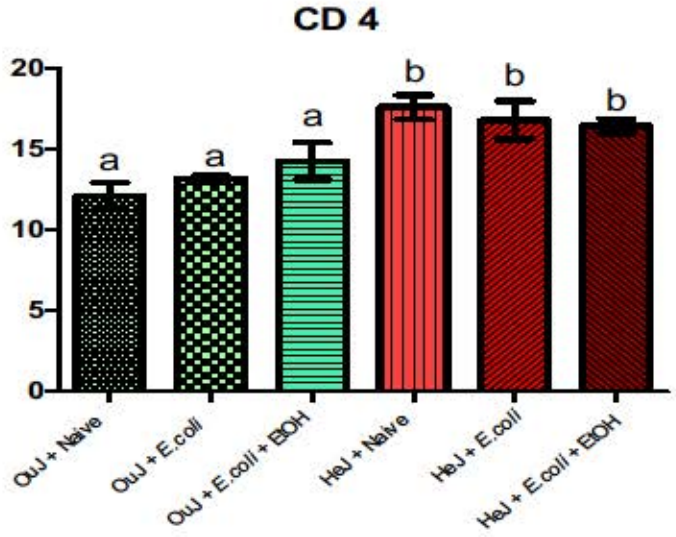
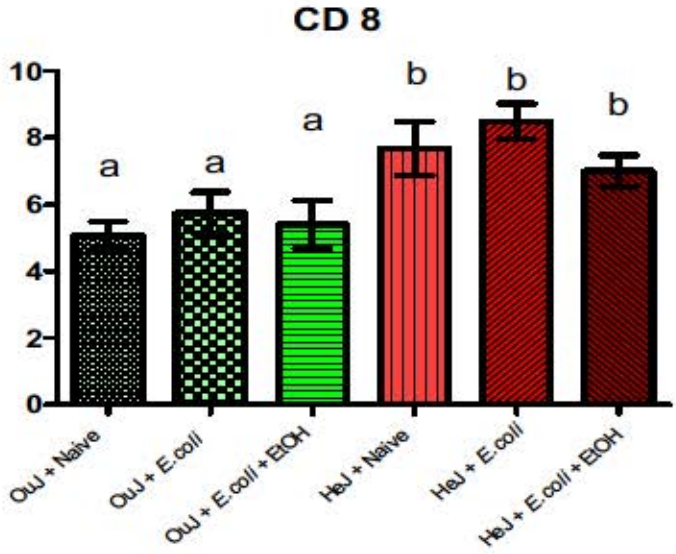
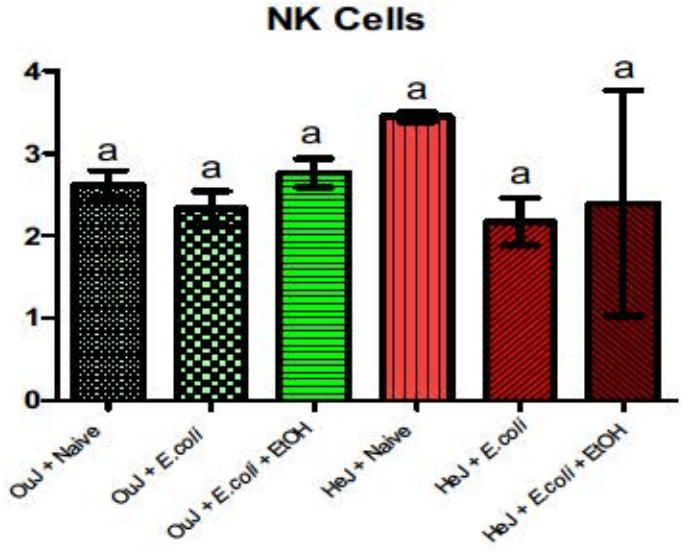


(a)



(b)

Figure 8 (a)-(d) Represent the flow cytometry data obtained by carrying out flow cytometry analysis of the spleen samples from both the control as well as the mutant strains of the mice



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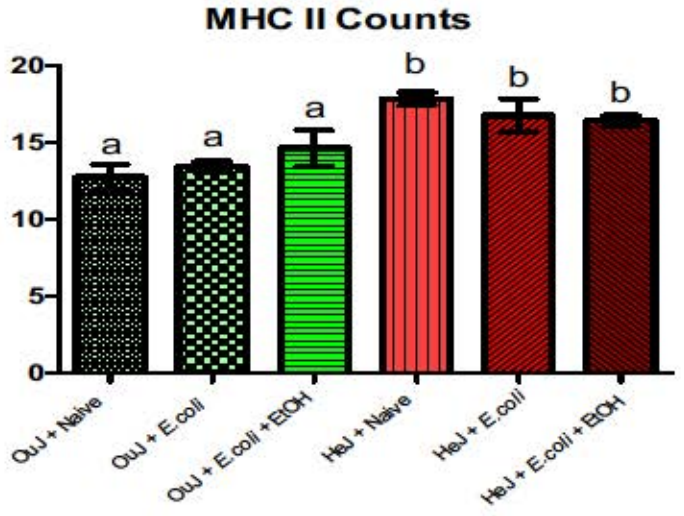

(d)

Figure 8 (continued)

CHAPTER IV

DISCUSSION

Alcohol consumption has long been recognized as a risk factor for infections. Previous studies in our laboratory and other laboratories have clearly demonstrated that acute ethanol treatment interferes with the innate immune response. The effects reported here occurred at relevant ethanol concentrations. The results presented here demonstrate that mice treated with ethanol and then challenged with non-pathogenic *E. coli* intraperitoneally show decreased clearance of bacteria by macrophages and decreased cell counts in both the peritoneal cavity and the spleen. The production of most pro-inflammatory cytokines and chemokines was also suppressed as was the attraction of neutrophils to the peritoneal cavity. These results indicate that ethanol inhibits the initial inflammatory response to *E. coli*, which, in turn, decreases the clearance of bacteria in the first few hours after the challenge.

Many studies have reported that ethanol inhibits TLR signaling (123, 124, 125, 126) and it seemed likely that this was involved in the decreased resistance to infection associated with acute ethanol exposure. However, the results presented in an earlier study done in our lab (55) indicate that survival was enhanced in the absence of fully functional TLR4, therefore inhibition of TLR4 signaling as the major mechanism by which ethanol suppresses resistance to sepsis seems unlikely. However, it is quite possible that

inhibition of TLR4 signaling either by ethanol or TLR4 absence does play an important role in decreased resistance to lower dosages of bacteria, as is the case with C3H/HeJ mice (127) and that clearance of higher dosages of bacteria could be delayed (128) even if ultimately effective. Studies have also shown that C3H/HeJ (TLR4 mutant) mice do not survive as well as wild type mice when a low dose of entero-pathogenic *E. coli* or a sub-lethal challenge dose of bacteria is administered (129, 127). However, when mice were treated with a greater lethal dose of bacteria, a much higher percentage of C3H/HeJ mutant mice survived than wild type mice (55). Similar results were also reported very recently (130), indicating that TLR4 mutant mice have increased resistance to a lethal outcome in *E. coli* sepsis caused by a high dosage of *E. coli*. Thus, it seems likely that the role of TLR4 in resistance to sepsis and lethality in sepsis depends on the initial challenge dose of bacteria.

In addition to the TLR4, there are various other receptors (130), both cytoplasmic as well as membrane bound that respond to LPS and there are other TLRs that respond to other components of gram negative bacteria. Therefore, it is quite possible that in the absence of TLR4, these other receptors mediate enough response to lead to bacterial clearance but not enough to over-produce the inflammatory mediators. The results presented here with regard to cytokine and chemokine production in TLR4 mutant and wild type mice support this idea.

The findings of the current study demonstrate that ethanol suppresses the production of most of the cytokines and chemokines and in fact, only wild-type mice produced more IL-10 when treated with ethanol and also IL-17 production was not suppressed in the mutant mice when treated with ethanol. These results are in agreement

with an earlier study by our laboratory (131) and other studies (132, 25) which have also shown that ethanol suppresses cytokine and chemokine induction. It has also been established that response of macrophages to whole gram negative bacteria involves responses through several TLRs that are also induced by gram-positive bacteria and a response through TLR4 induced by LPS of the gram-negative bacteria (133). Thus it is not surprising that ethanol, which suppresses responses through most or all TLRs, would suppress resistance to the gram-negative bacterium *E. coli*. These results support the idea that the suppression of pro-inflammatory cytokines, the increase in IL-10 and probably other changes are sufficient to diminish host resistance. This is further supported by the observation that the effects of ethanol on cytokine concentrations in mice challenged with *E. coli* were similar to the effects of ethanol on LPS-induced cytokine production (134). In a previous recent study done in our lab (55), it has been shown that mice did not begin to die until about 20 h approximately after treatment, it seems possible that ethanol induced decrease in pro-inflammatory cytokines and chemokines and the substantial increase in IL-10 concentration allow uncontrollable replication of *E. coli* until the effects of ethanol subside and fatal cytokine induced septic shock ensues.

The suppression of cytokines and chemokines was somewhat selective with regard to the amount of suppression but all pro-inflammatory cytokines tended to be decreased by ethanol treatment. The results from the cytokine and chemokine study also showed various patterns in response to the different treatments by both the control as well as the mutant mice. While a group of cytokines (IFN- , IL-1α, IL-6, IL-9, IL-13, IL-15, IL-12p40, IL-12p70, MIP-2, M-CSF, G-CSF, MIG, MCP1, LIX, IP-10) was suppressed by the treatment of ethanol in both the strains of mice, it didn't seem like TLR4 absence

had any major effect in their production. In fact, the number of these cytokines and chemokines produced by both the strains of mice for similar treatments doesn't look too different.

The other group of cytokines and chemokines showing similar trends includes the IL-1β, TNF-α, Eotaxin, GM-CSF, RANTES, MIP-1α, MIP-1β and LIF. While the production of these cytokines and chemokines showed huge suppression due to ethanol treatment in both the wild-type and mutant mice, it looks like the absence of TLR4 also played a role because the production of cytokines and chemokines in the mutant strain of mice was very low compared to the wild-type mice for both the treatments.

While the IL-10 production in wild-type mice was enhanced due to the ethanol treatment, its production in the mutant mice was very low and there wasn't any significant difference in its production due to the ethanol treatment. It is interesting to note that the enhancement of LPS-induced IL-10 production by ethanol has been previously reported using human subjects also (134). Thus it seems likely that this is a general phenomenon. For the IL-17 cytokines, while its production was very significantly decreased due to the ethanol treatment in the wild-type mice, the mutant mice showed an opposite effect, i.e., the ethanol treated mice produced more IL-17 cytokines than the non-ethanol mice, even though the difference was not significant in nature.

Considering some cytokines like IL-6 and IL-12 generally enhance immunity and IL-10 is suppressive in most systems, the net result of ethanol induced changes in these parameters would probably be immunosuppressive. These results are generally consistent with reports from other studies indicating suppression of proinflammatory cytokines (135) and increased IL-10 (136) associated with alcohol consumption (137).

It is well recognized that IL-10 can inhibit protective immune response to infections (138). It has been shown that the trauma, burn, and major surgery-induced immuno-depression, which predispose to infectious complications, is related to IL-10 over-expression (139, 140, 141). It has also been shown that IL-10 controls inflammatory processes by suppressing the expression of proinflammatory cytokines, chemokines, adhesion molecules, as well as antigen-presenting and co-stimulatory molecules in monocytes/macrophages, neutrophils, and T cells (138). Considering the ability of IL-10 to inhibit monocyte function, it is likely that elevated IL-10 levels contribute to the disturbed cellular immune response observed after acute alcohol treatment. As all of the inflammatory proteins are transcriptionally controlled by NF-κB it has been suggested that IL-10 may exert a significant part of its anti-inflammatory properties by inhibiting this transcription factor (142, 143).

On the other hand, IL-10 has also protective effects in infection because it prevents an uncontrolled inflammatory response to infectious triggers. IL-10-deficient mice have been observed to show a prolonged inflammatory response to acute *Pseudomonas* challenge resulting in neutrophil accumulation in the lung. This observation suggests that IL-10 deficiency might contribute to prolonged inflammatory responses early in cystic fibrosis, a lung disease that is characterized by a neutrophilic infiltrate that is excessive relative to the burden of infection (144). Overexpression of IL-10 prevents mice from endotoxin or bacteria-induced septic shock whereas lack of IL-10 increases the susceptibility to toxin-related shock (138, 145). IL-10 also protects against experimental group B streptococcal arthritis (146). Similar protective properties of IL-10 were observed for gastrointestinal helminth infections (147). Because IL-10 expresses

potent immuno-modulatory properties it can modulate the course of infections. The main target of IL-10 is macrophages, and these cells play a central role in infections, as a target for pathogens and in the activation of both specific and innate immune response.

Many studies have demonstrated that IL-17 appears to be an important mediator of inflammation, especially in neutrophil-dominated responses to bacterial challenge (148). This connection is intriguing given that expression of IL-17 is restricted to memory T cells, which are associated with an adaptive immune response, while neutrophils are viewed primarily as mediators of innate immunity. It has been hypothesized that by secreting IL-17, which subsequently induces chemokines and granulopoietic factors, memory T cells may enhance faster and more effective recruitment of neutrophils (149). In this respect IL-17 may serve as a modulator of early immune responses to pathogens, and as such may be an important element of host defense. On the other hand, the overproduction of IL-17 may aggravate inflammatory reactions and contribute to tissue injury. In such situations IL-17 may be viewed as a potential target for therapeutic intervention, and this approach is now intensively being explored by the pharmaceutical industry.

Overall, the results obtained here demonstrate that ethanol treated mice exhibit decreased clearance of bacteria and produce lesser amounts of most pro-inflammatory cytokines. This suggests that ethanol inhibits the initial inflammatory response to *E. coli* and this leads to decreased clearance of bacteria. Large number of macrophages in the peritoneal fluid indicates the decreased attraction of neutrophils to the peritoneal cavity and decreased clearance of bacteria by macrophages and neutrophils in the peritoneal cavity, hence the increased mortality.

TLR4 is dispensable for survival in *E. coli* sepsis. TLR4 contributes to lethality in wild-type mice. A hypo-functional TLR4 allows survival in mice. LPS stimulates a unique and critical pathway of innate immune responses that is independent of TLR4 and results in early neutrophil infiltration and enhanced bacterial clearance. In summary, cytokines are key modulators of inflammation. They participate in acute and chronic inflammation in a complex network of interactions. Several cytokines exhibit some redundancy in function and share overlapping properties as well as subunits of their cell surface receptors. Better understanding of the pathways regulated by cytokines will allow the identification and/or development of agents for improved modulation of the inflammatory response for the treatment of autoimmune and other infectious diseases.

In conclusion, attraction of TLR4 signaling pathways by LPS is a critical upstream event in the pathogenesis of gram-negative sepsis making TLR4 an attractive target for novel antisepsis therapy. Recent studies have shown that anti-TLR4 antibodies inhibited intracellular signaling, markedly reduced cytokine production and protected mice from lethal endotoxic shock and *E. coli* sepsis when administered in a prophylactic and therapeutic manner upto 13 h after the onset of bacterial sepsis (150). These experimental data provide strong support to the concept of TLR4 targeted therapy for gram-negative sepsis (151). At a time when most anti-sepsis clinical trials have frustratingly yielded negative results, these experimental data provide strong support to the TLR4-targeted therapy currently underway in patients with gram-negative sepsis.

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