ESTROGEN REGULATION OF TUMOR NECROSIS FACTOR ALPHA DURING DR^{+} *ESCHERICHIA COLI* UROPATHOGENESIS

By

BRITTANY ROCHELLE BOLT

Bachelor of Science in Biology

Minor: Chemistry, Spanish

Missouri State University

Springfield, MO

2005

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
July, 2010

ESTROGEN REGULATION OF TUMOR NECROSIS FACTOR ALPHA DURING DR^{+} *ESCHERICHIA COLI* UROPATHOGENESIS

Thesis Approved:

Dr. Rashmi Kaul

Thesis Adviser

Dr. Alexander Rouch

Dr. Kathleen Curtis

Dr. Mark E. Payton

Dean of the Graduate College

ACKNOWLEDGMENTS

I would first like to thank my Savior for His guiding mercies. Thanks to He who has taken me through the challenges along the road to attaining an advance degree in science.

I would like to acknowledge the members of advisory committee for their patient and committed support and guidance. To Dr. Rashmi Kaul my research advisor, I would like to say thanks for your invaluable investment into my future. I am thankful for the opportunity to gain my Master of Science degree in your lab has provided for me. My wish for you is that you will have blessings and peace always-Thank you! To Dr. Al. Rouch and Dr. Kath Curtis I say thank you. My advisory committee has provided strong legs to stand on as I reach for my goals. Im especially thankful for the times I was the benefit of your advice, even when it did not pertain to science. Thanks for your vote of confidence and your tutelage on matters of life.

To my lab members, Dr. Rashmi Singh and Dr. Senait Assefa, thanks for the countless hours you have spent investing into my future. May all your goals be achieved and may your accomplishments bring you great success and happiness.

To my family I say thanks and take a bow, for without you this achievement would not have been possible. Errol Bolt, Carmen Bolt, Brianna Bushnell, Bradford Bolt and Rick Bushnell, merely names on a sheet of paper but in truth they are the fabric which holds me together. I'm thankful that though I am in the United States, and Jamaica is miles away, your love is always tangible. Mommy and Daddy, thanks for teaching me about my self-worth and the truth of where it lies.

I would like to acknowledge the Oklahoma State University, Center for Health Sciences Department of Biomedical Sciences, for providing a stimulating and efficient learning environment.

TABLE OF CONTENTS

Chapter Page

LIST OF TABLES

Table Page

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Urinary Tract Infections (UTI) are among the most common diagnoses in primary care, hospitals, and extended care facilities. UTIs account for the use of 6 billon health care dollars in treatment and management, and cause 8 million patient visits, ultimately resulting in over 100,000 hospitalizations per annum (Drekonja, and Johnson. 2008; Stamm 2001). In over 80% of UTIs, the causative agent is a strain of uropathogenic *Escherichia coli (UPEC;* Chassin *et al.*, 2006; Mulvey, 2000*).* Drug resistant bacterial strains have added to the complications of UTIs, resulting in increased frequency of recurrent UTIs (Blanogo, 2010; Drekonja and Johnson, 2008). Originating in the colon, these especially virulent uropathogens express adhesive proteins such as P pilli, Type 1 pilli, and Dr adhesins which mediate adherence to the epithelial cell surface. Specifically, Dr fimbriae are associated with a 2 fold increased risk of recurrent UTIs. Invasion of the cell by UPECs containing Dr fimbriae (Dr+ *E. coli)* have been shown to cause chronic pyelonephritis in the C3H/HeJ mouse model (Kaul *et al., 1999; F*oxman, *et al.*, 1995; Nowicki *et al.*, 2001).

Dr fimbriae bind to uroepithelial cells of the urinary tract through recognition of decay acceleration factor (DAF), also known as complement regulatory protein, CD55 (Nowicki 2001). DAF, a membrane associated glycoprotein, interacts with Type IV collagen, which is a colonization receptor. This interaction facilitates the invasion of Dr+ *E.coli* into the cell, causing the infection and inflammation observed in UTIs. Previous experiments in our lab have demonstrated that this interaction is hormonally regulated (Selverangan, 2004; Singh *et al.*, 2010)

Association with Toll like receptor 4 (TLR) is essential for the initiation of host responses due to invasion of the cell by UPECs. TLR4 binds to lipopolysaccharide (LPS) of the gram negative UPECs and is therefore activated to stimulate the production of pro-inflammatory cytokines (Vandewalle, 2008).

UTIs can affect both men and women; however, they are more prevalent in females. Approximately 50% of adult women report experiencing one or more UTI per annum and some women develop a history of repeated infections (Drekonja, and Johnson, 2008). One of the most significant clinical issues of UTIs is recurrence, the mechanism of which is only partially understood. The incidence of UTIs in females is contingent upon age and stage of menstrual cycle. In particular, postmenopausal women demonstrate drastic decreases in estrogen and maintain high incidences of UTIs. This observation, coupled with the fact that UTI occurrence varies with stage of menstrual cycle, suggests hormonal regulation (Curran *et al.*, 2007, Straub 2007).

Clinical trials using estrogen hormone-replacement therapy have demonstrated decreased incidence in UTIs in postmenopausal women and established estrogen-replacement guidelines for the prevention of UTI (Dwyer, 2002). Although, there are studies suggesting that estrogen increases the risk of UTI (Orander, 1992; Curran *et al.*, 2007). It is widely agreed that estrogen has a significant role in protection against infection (Styrt, 1991). Estrogen may act on the host,

the pathogen, or the host-pathogen system as a means of mediating immunity. Estrogen has a wide range of action, via estrogen receptor (ER) α or ER β to regulate gene transcription (Straub, 2007). In the kidney, the predominant receptor subtype is ERα (Jelinsky *et al.*, 2003). Although it has been established that estrogen exerts both pro-inflammatory and anti-inflammatory effects during pathogenesis, one of the crucial ways in which estrogen regulates the immune system is via pro-inflammatory proteins (Fahey, 2008).

Tumor Necrosis Factor alpha (TNF-α) is an instrumental factor in mounting the innate and adaptive immune response and is one of the most important cytokines in resolving UTI pathogenesis. Estrogen effects on TNF-α have been extensively studied. *In vitro* studies suggest that estrogen suppresses the production of TNF-α in numerous cell types and pathologies (Lambert, 2004). However, estrogen-mediated TNF-α production is increased in acute pyelonephritic UTIs (Görgoze *et al.*, 2005). This speaks to the dichotomous and cell-specific action of TNF-α, and demands further inquiry into the mechanism of action.

UTIs are a far reaching significant clinical problem for which treatment options are poorly understood. Estrogen-mediated protection against UTIs represents a plausible option for patient treatment. As a result, we have developed an *in vitro* model of the ascending UTI using murine inner medullary collecting duct cells of the kidney (mIMCD-3). We hypothesize estrogen modulates Dr+*E.coli* invasion in mIMCD3 cells by regulating TNF-α production and affecting expression of DAF. Therefore, the current study was conducted to investigate the role of estrogen in modulating TNF-α response during Dr+*E.coli* infection in mIMCD-3 cells. The long term goal of this study is to identify and characterize the cellular and molecular mechanisms through which estrogen or estrogen receptors may be modulating the onset and severity of UTIs. This information will contribute to the understanding of UTI pathogenesis and the identification of biomarkers with hopes of leading to effective treatment for the incidence of UTIs.

CHAPTER II

REVIEW OF LETERATURE

Urinary Tract Infections

It is estimated that 150 million UTIs occur globally per annum, resulting in more than 6 million dollars of indirect health care expenditure (Stamm, 2001). In the United States, UTIs account for 8 million patient visits per year, 1 million of which are emergency department visits. Collectively, these visits incur billions of dollars for management, and result in over 100,000 hospitalizations. The incidence of UTIs occurs predominantly among women, demonstrated by the fact that nearly 1 in 3 women will be diagnosed and require antimicrobial therapy by the age of 24 years (Foxman, 2003). Additionally, the prevalence of UTIs in the female population varies with age and menstrual cycle, confirming the possibility of hormonal control via estrogen (Sobieszczyk, 2008).

UTIs may involve either the lower urinary tract, or both upper and lower urinary tract (Figure 1). The term cystitis describes infection in the lower urinary tract and presents as dysuria and suprapubic tenderness with urinary frequency and urgency (Nicolle, 2008). Pyelonephritis describes urinary tract infection ascending toward the kidney. It is a more severe diagnosis, presenting with dysuria, abdominal pain and often systemic symptoms including, but not limited to, fever, rigors, headache, vomiting and delirium (Ramakrishnan, 2005).

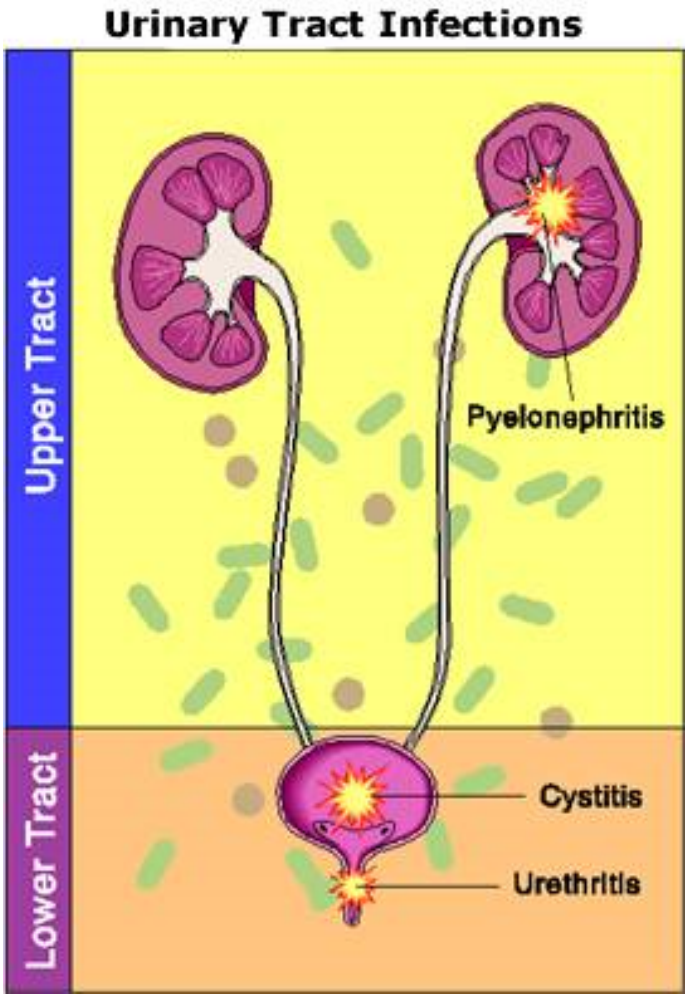

Figure 1: Location of upper and lower urinary tract infection. Adapted from Supplementnews.org/progressive health.com.

Uropathogenic *E.coli*

Organisms that cause UTIs are derived primarily from the aerobic members of the fecal flora. 95% of uncomplicated cases of cystitis are caused by a single organism. In contrast, infections among hospitalized patients or those with structural abnormalities of the urinary tract derive from polymicrobial infections (Sobieszczyk, 2008).

The most common causative agent of UTIs are gram negative rods, 80% of which are members of the especially virulent family of Uropathogenic *E.coli* (UPEC) The majority of these uropathogens originate in the colon, invade the urethra, ascend to the bladder and then to the kidney. As UPECs invade the upper urinary tract in an ascending infection, they first come into contact with the cells of the inner medullary collecting duct (IMCD) in the kidney (Chassin *et al.*, 2006, Mulvey, 2002). Despite the fact that most all strains of *E.coli* can result in UTI, UPEC's are particularly virulent because of their ability to produce adhesive proteins on their cell surfaces called pilli, fimbriae, or adhesins. These proteins include P pilli, Type 1 pilli, and Dr adhesins, which mediate the adherence of bacterium to the cell lining of the bladder and upper urinary tract. P fimbriae (Figure 2) are associated with acute pyelonephritis, and initiate ceramide release and activation of signaling pathways (Wult *et al*.,2000). They require toll like receptor-4 (TLR-4) for binding and function, and elicit a strong proinflamatory response to lipopolysaccharide (LPS) challenge. (Hellund, 2001; Johnson, 1991). *E.coli* Type 1 fimbriae are associated with cystitis and mediate bladder cell invasion. Type 1 fimbriae require cluster of differentiation 14/ TLR4 (CD14/TLR4) interaction and signaling and cause the production of interleukin 6 (IL-6) via a LPS- dependent pathway (Curran, 2007). Of particular interest, *E.coli* Dr fimbriae are associated with increased risk of recurrent UTI and have been proven to cause experimental chronic pyelonephhritis (Goluszko *et al.*, 1997; Goluszko *et al.*, 2001; Nowicki *et al.*, 2001). The *E.coli* Dr fimbriae adhesin was originally cloned from the clinical pyelonephritis strain, *E. coli* IH11128. Biogenesis of Dr fimbriae requires the *dra*A gene as well as three others (*dra*C, *dra*D, *dra*E) (Norwicki, 2001). Dr adhesins of Dr+ *E.coli* have been shown to be essential for the induction of chronic pyelonephritis in the C3H/HeJ mouse model (Goluszko *et al.*, 1997; Nowicki *et al* 2001) They invade the uroepithelium using lipid rafts and microtubule networks and subsequently bind to the cells of the uroepithelium via recognition of type IV collagen and decay-accelerating factor (DAF), also known as CD55 (Curran, 2007).

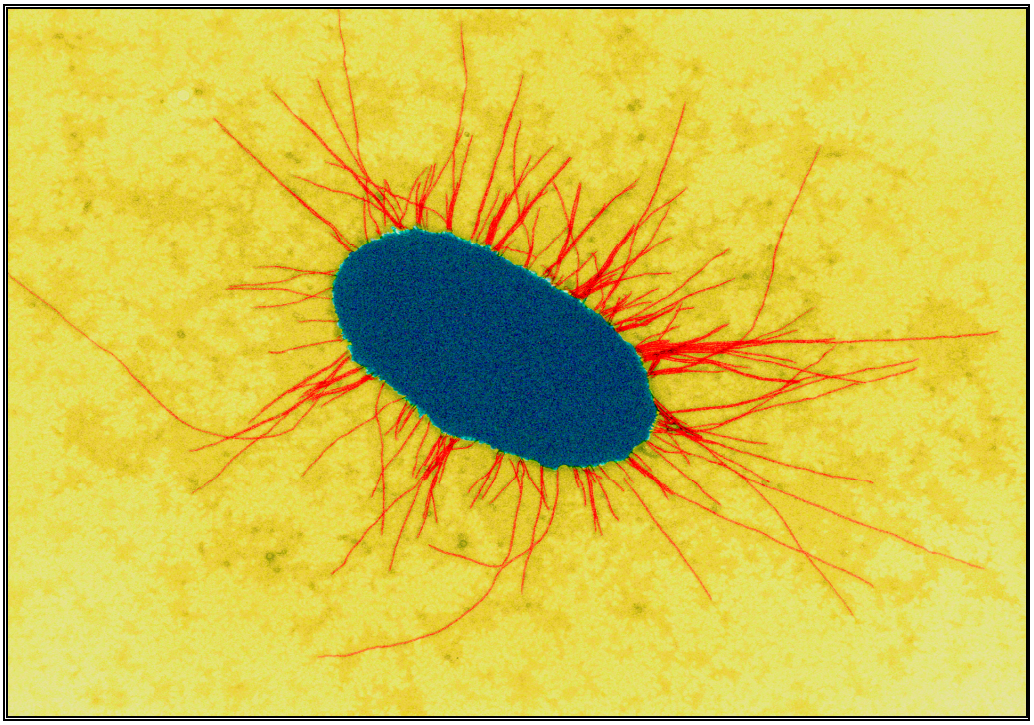

Figure 2: *Escherichia coli* demonstrating fimbriae Image adapted from Dennis Kunkel Microscopy, Inc.

DAF is a glycosyl-phosphatidyl-inositol protein bound to the cell membrane by a region of four consecutive extracellular short consensus repeats (SCR). The physiological function of DAF is to protect the host cell from the cytotoxic effects of complement activation. DAF has a wide tissue distribution, which includes epithelial surfaces of the gastrointestinal mucosa, exocrine glands, renal pelvis, ureter, bladder, cervix, and uterine mucosa. In the human endometrial epithelium, DAF expression is dynamically regulated through the reproductive cycle, demonstrated by an increase in expression during pregnancy (Hasan, 2002; Fang, 2004; Selvarangan, 2004, Nowicki, *et al.*, 1990, Nowicki *et al.*, 1993; Lui, 2004, Mulvey, 2002). Dr+ *E. coli* recognizes DAF to adhere, invade, and colonize the kidney epithelium, and further binds to type IV collagen in the epithelial basement membrane (Selvarangan, 2004).

Interaction of Dr+ *E.coli* with DAF and type IV collagen in the kidney leads to bacterial infection, inducing inflammation in renal tubules. Type IV collagen is found on the basement membrane of the renal interstitial cells and is a specialized form of extracellular matrix that underlies all epithelia and compartmentalized tissues. The adherence of bacteria to type IV collagen in the basement membrane of the epithelium facilitates renal persistence of Dr+ *E.coli* and the development of pyelonephritis (Selverangan, 2004).

The adherence of UPECs to the uroepithelium induces an inflammatory response via pathogen-specific virulence factors. The uroepithelium forms a physical barrier against microorganisms, which complements the passive shield of antimicrobial proteins. These proteins respond to invasion by activating immune cascades (Jahnukainen, 2003). Briefly, the inflammatory response progresses in three steps. First, bacteria simulate uroepithelial cells to produce pro-inflammatory mediators. Second, these chemokines and their chemokine receptors direct inflammatory cells to the site of infection. Third, the local inflammatory response determines the bacterial clearance or the extent of tissue damage that will result (Savanborg, 2001)

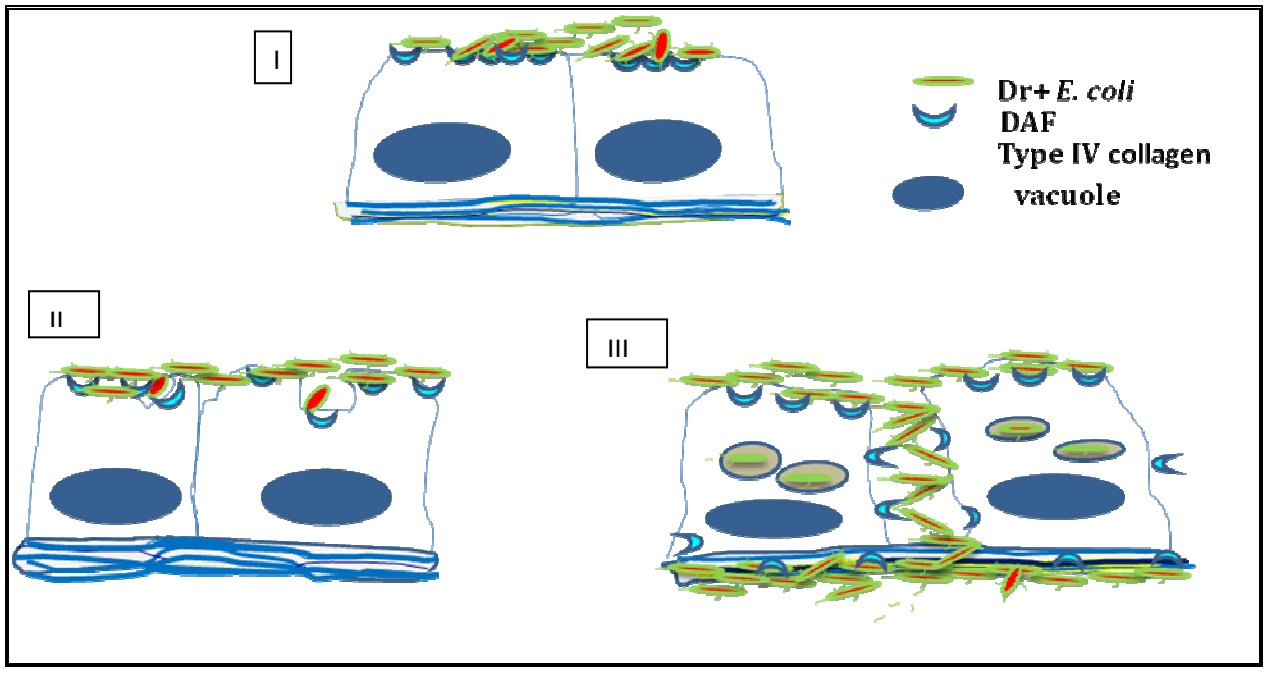

Figure 3: Mechanism of UPEC invasion in host cells during Dr+ *E.coli* uropathogenesis.

I. Attachment of Dr+ *E. coli* to DAF receptors on the apical surface of the epithelial membrane, resulting in bacterial colonization; **II.** DAF receptor clustering on the membrane and internalization by the phagocytic pathway due to the redistribution of cytoskeleton-associated factors and microtubules; initiation of internalization; **III**. Bacterial internalization into a vacuole within the host cell and attachment to type IV collagen in the basement membrane. Image adapted and modified from Singh *et al* 2010, information based on Mulvey, 2002.

UTI and Inflammation

Activation of the innate immune response in the urinary tract is dependent on the recognition of the UPEC pathogen-associated molecular patterns by pattern recognition receptors. UPEC pathogen-associated molecular pattern includes LPS, flagella, type 1 pilli, and P pilli that can act through TLR and nucleotide binding and oligomerization domain-like receptors (Rudick *et a*l., 2010; Zhang *et al.,* 2004; Fahey, 2008). The recognition of molecular patterns displayed on the surface of UPEC microorganisms by TLRs, and their subsequent activation, leads to the transcription of appropriate host-defense genes (Zhang *et al.,* 2004). TLRs play a vital part in the recognition of bacterial machinery. The renal tubule and epithelial cells express TLR 1, 2, 3, 4, 6 and 11. TLR 4 recognizes LPS, the main constituent of Gram-negative *E.coli*, and is a crucial interaction in mounting an immune response. TLR 11, expressed in mouse kidney and bladder epithelial cells (not in humans), is thought to aid in the recognition of UPEC pathogens (Vandewalle, 2008). Studies have demonstrated that UPECs invading the kidney specifically bind to the apical surface of collecting duct cells. The signaling pathways that are activated by UPECs in the collecting duct cells of LPS-sensitive C3H/HeOuJ and LPS-defective C3H/HeJ mice show that UPECs stimulate expression of pro-inflammatory mediators in the medullary collecting ducts via TLR4 pathways as a result of the activation of TNF (Vandewalle, 2008, Chassin *et al.,* 2007).

The UPEC-induced inflammatory response is a result of tissue damage caused by cell apoptosis, as well as neutrophil and macrophage activation. As reported by Rudick *et al.,* (2010), the local production of inflammatory chemokines results in the rapid recruitment of neutrophils into the bladder lumen, which in turn mediates bacterial clearance. The most potent of the local pro-inflammatory chemokines and cytokines implicated in the elicited response include, Il-6, IL-1β, IL-8, TNF-α, Nf-κB and others (Görgoze, 2005)

UTI and pro-inflammatory cytokine, TNF-α

Of the pro-inflammatory cytokines that control the response to UPECs, TNF-α is particularly important (Figure 4). Originally described as an agent of death in relation to tumor cells (Georgiadou, 2009), TNF-α is a cytokine with a host of actions. TNF-α is produced primarily by macrophages and other mononuclear phagocytes in the kidney (Ferreri, 2007). Although monocytes and macrophages are the main source of TNF-α, the local production of the cytokine stimulated by intrinsic renal cells is of greater significance in renal inflammation (Ernandez, 2009). TNF-α is known to stimulate chemokines and adhesion molecules, induce apoptosis, and activate the microbial system of phagocytes. TNF-α is implicated in renal inflammation and glomerular damage induced by immune complex deposition, as renal expression of TNF-α is up-regulated in both mice and human kidney infection (Ernandez, 2009) .

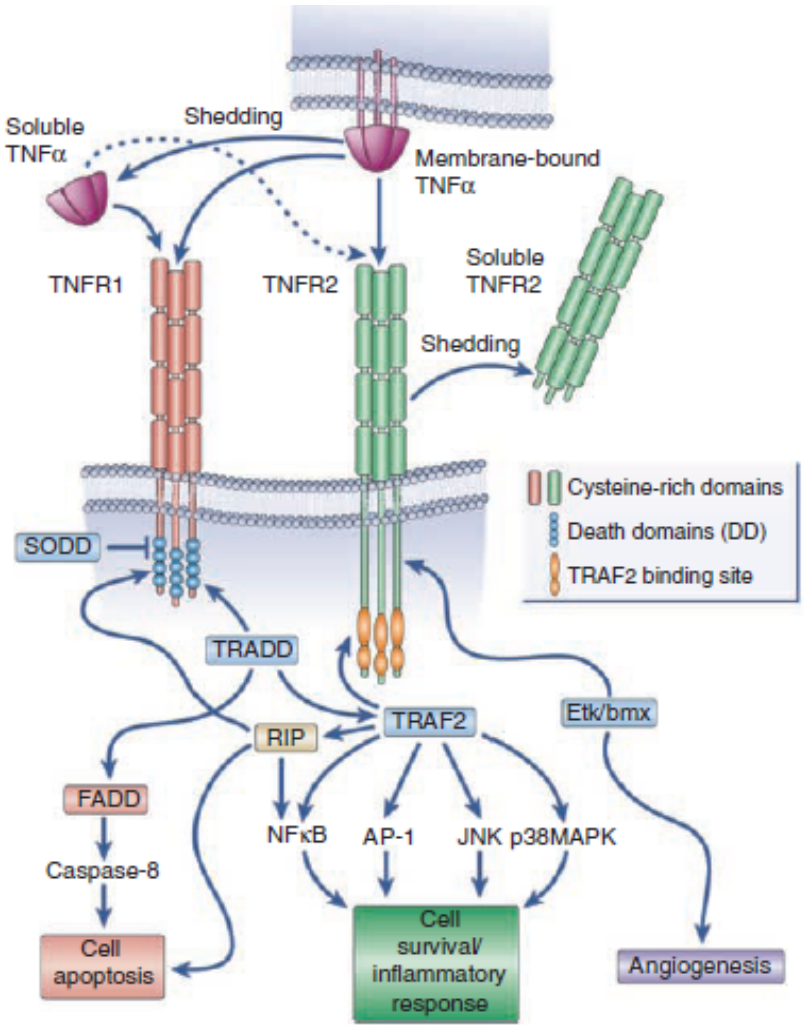

Figure 4: Intracellular signaling pathways induced by TNF-α. TNF-α induces the trimerization of the TNF receptor on the cell surface which causes the recruitment of adapter molecules to the receptor. The adaptor molecule-receptor complex pathways leads to activation of caspase 8 and apoptosis (the death pathway) or the activation of gene transcription factors AP-1via JNK and NF-κB (the survival pathway) (Ernandez, 2009).

Additionally, TNF-α activates the transcription factor NF-κB, which has been described as the master switch of the immune system (Male, 2006). Metcalfe *et al.* states that TNF-α is capable of up-regulating its own expression, in addition to that of other inflammatory mediators, as it induces renal fibrosis and apoptosis (Figure 4). Gene polymorphisms influence TNF-α production and inflammatory responses of the uroepithelium to UPEC challenge. Polymorphisms of the TLR gene are responsible for the delayed clearance of bacteria from the urinary tract, as well as the hyporesponsiveness to LPS and the resulting predisposition to septic shock (Jahnukainen 2005).

The excessive release of TNF-α may cause tissue damage as seen in LPS-induced renal failure due to TLR-4 mediated production of TNF-α. In contrast, blocking TNF-α improves renal function, while simultaneously aggravating the renal bacterial burden in experimental mouse models of renal failure. These findings speak further to the role of pro-inflammatory cytokines such as TNF-α in an immune response (Vandewalle, 2008). Importantly, levels of pro-inflammatory cytokines including TNF-α were increased in acute pyelonephritic UTIs, compared to levels in lower UTIs (Görgoze *et al.*, 2005).

In kidney disease, experimental data suggest that TNF-α exhibits both pro-inflammatory and immunosuppressive functions. These roles are relayed by two structurally distinct receptors, TNF-receptor 1(R1) and TNFR2. These receptors are only 28% homologous in their extracellular domain, and share no homology of intracellular regions (MacEwan, 2002). TNFR2 has a higher affinity for membrane bound TNF. Studies conducted by Ernandez in 2009 implicated TNFR2 in mediating inflammation in renal injury. Finally, considering that the collecting duct is the main site of adhesion for UPECs it is likely that hormones involved in controlling sodium and water reabsorption, particularly those that act in the collecting duct, could be involved in controlling the inflammatory response stimulated in the IMCD (Vandewalle, 2008).

Estrogen the immunomodulator

Sex steroids play an important role during the inflammatory response by influenceing injury-induced cytokine production (Metcalfe, 2006). Estrogen is a steroid hormone which plays a vital role in reproduction, growth, development, and maintenance of numerous tissues. The physiological effects of estrogens are mediated through one of two estrogen receptors (ER), ERα and ERβ, both of which belong to the superfamily of nuclear receptors. ERs integrate multiple signals from ligands and intracellular signaling pathways to perform functions in the nucleus and cytosol (Moggs, 2001). ERα and ERβ are products of different genes and elicit tissue and cell type specific estrogen-mediated activity. Additionally, each receptor has different transcriptional activities in certain ligand, cell type, or promoter contexts (Mathews, 2003). The ligand binding domains of each receptor exhibit an affinity for endogenous estrogen, 17β-estradiol (E2). However, ERα and ERβ demonstrate different affinities for some natural compounds, as well as for novel subtype-specific ligands, demonstrating that the ERs are similar, but maintain unique roles in estrogen actions (Kuiper, 1997, Mathews, 2003). ERα localizes on the uterus, liver, kidney and heart, while ERβ is expressed primarily on the ovary, prostate, lung, gastrointestinal tract, bladder, and hematopoietic and central nervous system. Both receptor subtypes are co-expressed in numerous tissues; however, both may not be expressed in the same cell type (Kuiper, 1997)

ER complexes affect gene expression through two main pathways, the classic genomic pathway and the non-genomic pathway. The classic pathway depends on direct interaction of estrogen with its receptor in the nucleus. These ER complexes then can directly mediate gene transcription. The action of ERα and ERβ are mediated by two transcription activation functions (AFs). AF-1 is the N-terminal ligand-independent activation function and AF-2 is the C-terminal

ligand-dependent activation function. These AFs mediate transcription and cell-promoter specificity (Nilssson *et al.*, 2001). The non-classical, non-genomic pathway is a rapid interaction that is dependent on the ability of estrogen to interact with steroid or non-steroid hormone receptors in the membrane (Lorenzo, 2003).

Estrogen mediated regulation of inflammation and inflammatory markers

Sex steroids in both in vivo and in vitro experimental models have been shown to influence immune function and inflammatory processes, although the mechanisms are poorly understood (Czlonkowska, 2005). Estrogens have both anti-inflammatory and pro-inflammatory functions. For instance, E2 enhances the production of proinflamatory interferon-γ and anti-inflammatory IL-10 in T cells (Fahey, JV 2008, Correale, 1998, Calabrese, 2001, Straub, 2007) ER-bound up or down-regulates the transcription of various genes by binding to estrogen response elements (EREs) of genes, or through interaction with transcription factors (Beato, 1989, Paech, et al 1997, Straub 2007). There is well documented evidence that post-menopausal women experience an increase in pro-inflammatory cytokines, as estrogen decreases (Kovas, 2005). However, estrogen has paradoxical effects on the immune system. The paradoxical immunomodulatory effect of estrogen is contingent on a number of factors. These factors include the source of immune stimulus, cell type involved during various phases of disease, the target organ, the stage of menstral cycle, the physiological concentration of estrogen, the variability of ER expression (which, in turn, varies with microenvironment and cell type), intracellular metabolism of estrogens, pro-inflammatory function, and the influence of sensory and sympathetic nervous systems (Straub, 2007).

The contention that estrogen regulates the immune system is supported by in vivo studies, in which protective functions fluctuate with the stage of the menstrual cycle (Straub, 2007). Additionally, numerous cytokines, chemokines and antimicrobials present in cervical-vaginal lavage vary with stage of menstrual cycle, whether measurements were taken during the early proliferative or late secretory phase of the menstrual cycle (Figure 5). Pro-inflammatory cytokine production varies with circulating levels of estrogen in females during pregnancy and menopause. For instance, the concentration of IL-8 and defensins are lowest at mid-cycle when ovulation occurs and E2 levels are elevated (Fahey, 2008). Studies have shown that high-dose estrogen treatment of C3H/HeJ mice resulted in increased bacterial infection rates in the kidney, regardless of the adhesin type (Stamm, 2007). Further studies have shown that estrogen can enhance the attachment of UPECs, challenging the claim that estrogen treatment is always protective (Curran, 2007).

Estrogen also functions as an immunomodulator by regulating the levels of IgG and IgA antibodies, which also are altered by the stage of the menstrual cycle. Immunoglobulins in human cervical mucus increase over the days leading up to ovulation and then decline to their lowest levels at other stages in the cycle (Beagley, 2003) Similarly, estrogen diminishes cell-mediated immunity in humans and rodents, demonstrated by the decrease in levels of natural killer (NK) cell activity in females compared to males. The activity of NK cells also decreases from the first to third trimester of pregnancy as estrogen levels demonstrate drastic concentration changes (Styrt 1991).

Finally, estrogen regulates immunity by suppressing or enhancing the expression of pro-inflammatory mediators; however, estrogen-mediated inflammatory disease is as a result of numerous factors (Straub 2007; Fahey, 2008).

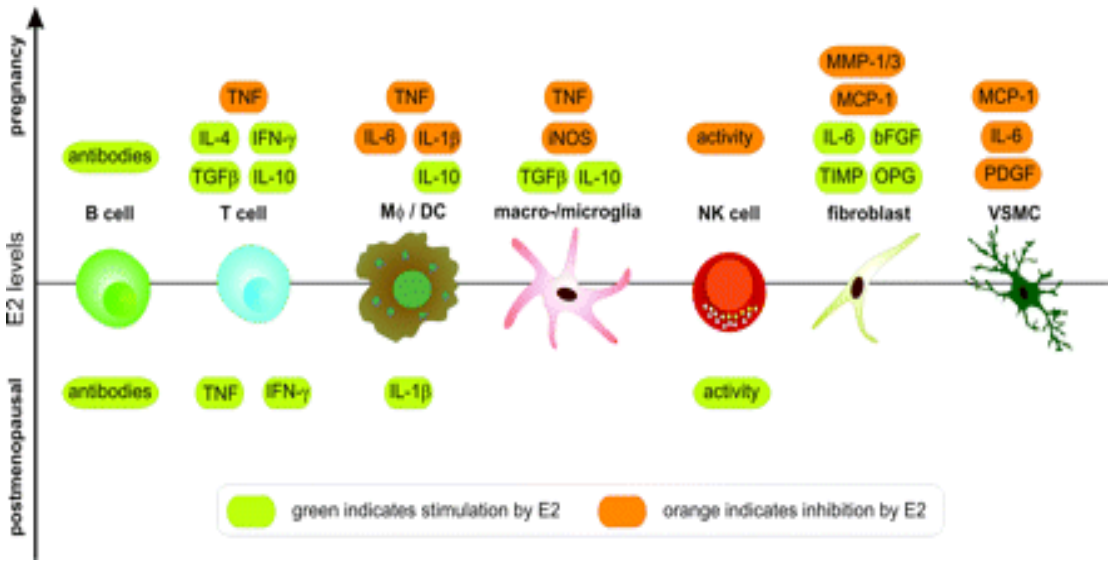

Figure 5: Demonstration of the pro-inflammatory and anti-inflammatory effects of estrogen on varying immune cell types throughout reproductive life. Various cell types demonstrate that at pregnancy levels, E2 effects on important pro-inflammatory pathways are inhibited. The orange color demonstrates the inhibition of TNF, IL-1β, IL-6, MCP-1, iNOS, production of MMPs, and the activity of natural killer cells. E2 at the same concentration stimulates anti-inflammatory effects such as Il-4, IL-10, TGFβ, TIMP and osteoprotegrin delineated by green. At lower E2 concentrations, E2 stimulates of TNF, IFN-γ, IL-1β and natural killer cell activity. Adapted from Straub, 2007.

TNF-α participates in innate immunity and adaptive immune responses. As a result of receptors present on virtually all cells, TNF-α is able to elicit diverse effects, including activating a variety of genes (Ferreri, 2006) Although TNF-α production is tightly regulated, this cytokine is widely reported to be elevated in postmenopausal women and ovariectomized (OVX) rats. This finding is consistent with *in vitro* experiments, and demonstrates the possibility of hormonal control of TNF-α production (Arenas, 2005; Kamada, 2001; Sites, 2002; Ferreri 2007; Metcalfe, 2006; Xing 2007; Huang, 2008).

The estrogen regulation of TNF-α has been extensively studied in numerous tissue types and pathologies. Estrogen demonstrates both up-regulation and down-regulation of TNF-α expression in various tissues in response to various agents of injury. For instance, E2 treatment causes profound up-regulation of TNF-α expression in the small intestine following trauma-hemorrhage (Chen, 2008), in human uterine epithelial cells following challenge by TLR3 agonist Poly(I:C) (Schaefer, 2005), in three week old mice challenged with oxazolone (OXA), a chemical allergen to the flank (Sakazaki, 2008), and in a renal ischemia/reperfusion model in rats (Wolfs, 2002). In contrast, E2 treatment also causes profound down-regulation of TNF-α expression as seen in vascular smooth muscle cells (Xing, 2007), in glial cells as a result of Alzheimer's disease pathology (Valles, 2009), in the dura due to spinal injury (Cuzzocrea, 2007), and in periodontal ligaments following LPS challenge.

Epidemiological and immunological evidence suggest that estrogen plays an important role in modulating bacterial invasion, susceptibility, and inflammation by mediating TNF-α expression and bacterial adherence (Ernandez and Mayadas, 2009, Mulvey, 2002). However, increased estrogen levels in pregnancy and decreased estrogen levels at menopause both enhance susceptibility to UTIs. Moreover, young women capable of reproducing have physiological levels of estrogen which play a vital role in decreased susceptibility to UTIs. Long term treatment with estrogen causes adverse effects in some groups (Straub, 2007). Additionally, TNF-α demonstrates pro-inflammatory and anti-inflammatory function in response to infection

(Ernandez and Mayadas, 2009). These conflicting findings make it difficult to predict the immunosuppressive effect of treatment with TNF- α or anti- TNF-α (which may lead to sepsis or autoimmunity). Therefore, it is necessary to further investigate the effects of estrogen on UTI susceptibility, invasion, and cytokine modulation at physiological levels. A better understanding of the role of E2 in regulating TNF-α during UTI pathogenesis may lead to novel means of treatment for the numbers who suffer from it.

CHAPTER III

RESEARCH DESIGN AND METHODOLOGY

Experimental cell line

The terminal inner medullary collecting duct (IMCD) serves a crucial role in the pathophysiological progression of a Urinary Tract Infection (UTI). The present study makes use of polarized epithelial murine IMCD-3 cell line (mIMCD-3) (ATCC CRL-2123^{TM}). This cell line was derived from the individual tubules of the terminal IMCD of mice transgenic for the early region of SV40 [Tg(SV40E)bri/7] (Rauchman *et al.,* 1993). This cell line, a gift from Dr. Hari Koul (University of Colorado at Denver and Health Science Center, Denver, CO), retains numerous characteristics of the terminal nephron segment. These characteristic include, but are not restricted to, high transepithelial resistance, inhibition of apical-to- basal sodium flux by amiloride, as well as the ability to grow in hypertonic medium common to the kidney medulla and lethal to most cell types (Rauchman *et al.,* 1993). The collecting duct system is the main site of adhesion of UPECs and, therefore, ideal for the investigation of experimental UTI (Vandewalle, 2008)

In the current experiments, *Dr+ Escherichia coli* strain IH11128 (O75;K5;H^{-}) was utilized to generate a model of an inflammatory UTI in mIMCD-3 cells to investigate the resulting uropathogenesis and the immunomodulatory effects of estrogen on TNF-α.

mIMCD-3 Cell Culture

mIMCD-3 cells were cultured in Dulbecco's modified Eagle Medium nutrient mixture F-12 (DMEM-F12; Invitrogen/Gibco) and supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals). Additionally, 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen) were added. The resulting confluent outgrowth of cells was observed on the surface of a T-25 cm^2 or T-75 cm^2 cell culture flask. Cells were then subcultured at a 1:4 ratio via trypsinization with 0.05% Trypsin, 0.053 mM EDTA (1X; Trypsin/EDTA) to attain 80-90% confluent monolayer. The culture monolayer was maintained in a 37°C humidified atmosphere (95%) containing 5% CO_2.

Initially, the mIMCD-3 cells were washed with 3-5 ml (depending on flask size) Ca^+ and Mg^+ free phosphate buffered saline (PBS) by gently rocking the flask for 30 seconds. The media was discarded and replaced with 2-3 ml of Trypsin/EDTA, and rocking was resumed for another 10 seconds. The trypsin-EDTA solution was discarded and the flask returned to the humidified atmosphere within the incubator as described above, for 10 minutes. DMEM-F12 media supplemented with 10% FBS and antibiotics (3 ml to 6 ml) was added to the culture flasks. After gentle agitation via pipette, the cell mixture was divided into two flasks. An additional 4.5 ml or 9.0 ml of DMEM-F12 media supplemented with 10% FBS and antibiotics was added to each T-25 cm^2 and T-75 cm^2 flask respectively.

Drug treatment of mIMCD-3 cells prior to bacterial invasion

In preparation for drug treatment, mIMCD-3 cells were washed with PBS. Surviving cells were counted via a haemocytometer and plated in 12-well plates at a density of 0.6 x 10^6 cells/ml/well. mIMCD-3 cells were cultured in medium containing DMEM-F12, 100 U/ml penicillin, 100 µg/ml streptomycin, and 5% charcoal stripped FBS (VWR). The cells were returned to incubation for 18 to 24 hours to allow for growth and adherence to the plate surface, after which the cell monolayer was treated with various drugs. Specifically, the mIMCD-3 cell monolayer was treated with 10 nM E2 (Sigma-Aldrich) or co-treated with 10 nM E2 and 10 µM ICI 182,780 (Tocris Bioscience). Drug compounds were diluted to desired concentrations by adding serum free media. In the same vein, control conditions were established by adding serum free media and omitting drugs. The cells were then returned to a 37°C humidified atmosphere containing 5% CO_2 for 24 hours prior to the introduction of Dr^+ *E. coli* strain IH11128

Induction of UTI in mIMCD-3 cells

Dr^+ E. coli strain IH11128 (O75;K5;H^-) was received from Dr. Bogdan Nowicki (University of Texas Medical Branch at Galveston, Texas). The pure culture was maintained in our laboratory under cryoprotective conditions at -80 ° C. Dr^+ E. coli strain IH11128 is a clinical isolate obtained from a female patient diagnosed with asymptomatic pyelonephritis (Nowicki, 1996) and has previously been used to induce uropathogenic inflammation (Kaul *et al.*, 1999; Curran, 2007).

Induction of experimental UTI in mIMCD-3 cells was accomplished by the introducing fresh Dr+ *E.coli* isolates in suspension as previously described (Kaul *et al.*, 1999). Several loops of bacterial inocula grown overnight at 37°C on Luria agar (LA) plates were suspended in sterile,

serum-free, antibiotic-free DMEM-F12 media; the optical density of the bacterial suspension was 0.5 at 600 nm.

The mIMCD-3cell monolayer was overlaid with 100 µl of Dr+ *E. coli* in suspension, and an equal volume of treated (10nM E2, 10nM E2 and 10µM ICI 182 780) or untreated medium added to each well. The simulated cell invasion and infection was allowed to proceed by incubating for 2 or 8 hours, in the previous humidified environment. External bacteria were then removed by two washes with sterile PBS.

Two hour infection bacterial invasion

After 2 hours, plates were removed from the humidified environment. The supernatant was collected for ELISA studies and stored in a cryo-protective environment at -80°C. The cell monolayer was harvested for RNA isolation and subsequent quantitative real time reverse transcriptase-polymerase chain reaction (RT-PCR).

8 hour bacterial infection invasion

A second group of plates was removed from the humidified environment after 2 hours. Supernatant containing extracellular Dr+ *E. coli* bacteria was removed and collected for ELISA studies, this was done to minimize the possibility of increase in bacterial cell division that could artificially increase the bacterial count of the supernatant at 8 hours. These plates were then returned to humidified incubation for an additional 6 hours. Collected supernatant, harvested 2 hours and 8 hours post infection were stored in a cryo-protective environment at -80°C and used for ELISA studies. The cell monolayer was harvested for RNA isolation and subsequent quantitative RT-PCR.

. **RNA isolation and cDNA synthesis**

i) Homogenization of cell extracts

Total RNA isolation was accomplished via the use of Trizol reagent (Invitrogen) as instructed by the manufacturer. The cells were lysed directly in the 12 well culture plate by adding of 333 µl of Trizol reagent and passing the cell lysate through a pipette tip repeatedly. Finally, the lysate was added to separate labeled sample tubes.

ii) Phase separation of cell lysate

Homogenized mIMCD-3 cells were incubated for 5 minutes at room temperature to disassociate nuclear proteins, after which 200 µl of chloroform was added to the cells. The sample tubes were then agitated vigorously for 15 seconds followed by centrifugation at a maximum of 12,000 g for 15 minutes at 4° C. After centrifugation, the homogenized cell lysate separates into two layers, an upper colorless aqueous phase and a lower red phenol chloroform phase. The aqueous phase retains RNA and was transferred 100 µl at a time into separate RNAse free tubes until the entire volume from each sample tube was transferred.

iii)RNA precipitation

700µL of isopropyl alcohol was added to each RNAse free aqueous phase-containing tube. The samples were incubated at 30°C for 10 minutes, then centrifuged at 12,000 g (4° C) for 10 minute until a jelly-like pellet was seen in the sample tube.

iv) RNA washing and re-dissolving

The isopropyl alcohol-containing supernatant was removed and the pellet then was washed with 75% ethanol (12.5 ml of RNase-free water and 37.5 ml of ethanol). Up to 1000 µl of 75% ethanol was added to the pellet and mixed by way of vortex and centrifugation at 8,000 Xg for 5 minutes at 4° C, after which the supernatant was discarded. 1000 µl of 75% ethanol was again added to the pellet and mixed by way of vortex and centrifugation at 8,000 Xg for 7 minutes at 4° C. Ultimately, final remnants of supernatant were carefully removed by pipetting.

After washing, the pellet was allowed to dry completely for 20-30 minutes by ventilating and inverting sample tubes.

RNA was re-dissolved in 50 µl of RNase-free. Solution was passed through a pipette tip multiple times followed by a 10 minute incubation period at 55° C – 60° C.

vi) RNA quantization and Integrity verification

RNA was quantified using NanoDrop spectrophotometer technology as per the manufacturer's instructions (ND-1000 V 3.3.1). The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA and RNA. Additionally, the 260 nm : 230 nm ratio of absorbance is a secondary measure of nucleic purity. Values for the 260/280 ratio and 260/230 ratio should be approximately 2.00, with values of 1.8-2.0 accepted for either ratio Values outside of the acceptable range suggested protein contamination were corrected by RNA purification with RNAeasy Mini Kit (Qiagen).

RNA integrity was verified on 1% Agarose (Invitrogen) denaturing gel. NorthernMax Gly 10X gel prep/ running buffer (Ambicon), Millenium marker and Glyoxal Sample load Dye (Ambicon) were used. 1µg quantities of RNA were loaded onto the agarose gel to ensure proper visualization.

vii) DNase Treatment and cDNA Synthesis

DNase treatment was performed on 10 µg of RNA with 2U of enzyme in a 50 µl system following kit instructions (Ambicon). EDTA was employed to stop the reaction via the chelation of metals.

cDNA synthesis employed the total RNA from each sample, which underwent reverse transcription. Our lab makes use of high capacity cDNA kit. Manufacturer provided 2X reverse transcription master mix was constituted from the contents of the high capacity cDNA kit. RNA samples were then added, and the mixture placed in a thermal cycle (Table1) to perform cDNA synthesis, as per the instructions from Applied Biosystem.

Quantitative real-time Reverse Transcriptase–polymerase chain reaction (real-time RT-PCR)

Quantitative RT-PCR is a technique that provides amplified DNA for the detection of relative or absolute DNA content. RT-PCR is driven by a polymerase enzyme and is dependent on thermal cycling which, in turn, controls primer binding and polymerase activity (http://www.primerdesign.co.uk). Fluorescing dyes that bind to double stranded DNA are used to monitor PCR in real time. In our lab, fluorescing SYBR green serves as a detector for cycle threshold (Ct). SYBR green is a fluorogenic minor groove binding dye which binds to double stranded DNA formed in PCR, thereby quantifying it.

An ABI StepOne Real Time PCR System (Applied Biosystems) was used to perform real time PCR. PCR was conducted using the following cycle parameters: holding stage- 95° C for 10 min (1 cycle), then 95 ° C for15 sec, 60° C for 1minute (40 cycles); Melt curve- 95°C for15 sec, 60 ° C , 95° C for 15 sec (1 cycle). SYBR green chemistry was used to obtain data from the cDNA samples introduced into the system using Power SYBR green master mix (Promega), Go Taq qPCR master mix (Promega) and cDNA templates. TNF-α and DAF gene expression were measured (Table 1). Primers were received from realtimeprimes.com in 50 µM concentrations and were then reconstituted to a working dilution of 1 µM. Primers contained forward and reverse primers in each sample. The internal control was an endogenous primer, Peptidylprolyl Isomerase A (PPIA), which allowed us to normalize the mRNA target to total RNA, thereby correcting for differences in the amount of total RNA.

Samples were run in duplicate with a hot start. A melt curve was performed and each sample was quantified using SYBR green quantitative PCR machines and chemistry. The total content of double stranded DNA in each well at each cycle produced the Ct value.

PCR data from each treatment group was analyzed based on values normalized to the measured expression of PPIA. Ct values were calculated based on the normalized values. Values were calculated as ΔCt= Ct target-Ct PPIA and via interpretation of relative gene expression data (RQ) calculated as follows; $2^{-\Delta CT}$. Mean RQ values for each sample group were used for statistical analysis.

Table 1: Primers for real-time RT-PCR (realtimeprimers.com)

Primer	Primer Sequence	Gene accession	Amplicon Size	Recognition region genome
DAF/CD55	Fp:TTCTCCCTTTGCTACGTCAC Rp: TCGCTTTGTCAACGACTTC	NM_010016	247bp	246-492
PPIA	Fp:AGCTCTGAGCACTGGAGAGA Rp: GCCAGGACCTGTATGCTTTA	AK028210	178bp	155-332
TNF-α	Fp: CCCACTCTGACCCCTTTACT Rp:TTTGAGTCCTTGATGGTGGT	NM_013693	201bp	899-1-099

Quantitative Enzyme Linked Immunosorbent Assay (ELISA)

TNF-α protein present in each treatment group was detected and quantified using mouse TNF-α ELISA Ready-SET-Go! Kits from eBiosicence. Kits contain capture antibody, polyclonal detection antibody, Avidin enzyme, assay diluent, substrate, ELISPOT coating buffer, and standard in the form of Recombinant TNF-α (1 ug/ml). The standard curve range was 8-1000 pg/ml. Plates were read via spectrophotometer and recorded with softmaxPro software.

Statistical Analysis

The data collected from all experiments were analyzed with GraphPad Prism 4 software. One way ANOVAs was followed by Tukey's post hoc testing when significant effects were found. P-values less than 0.05 were taken as indicative of statistical significance.

.

CHAPTER IV

FINDINGS

TNF-α mRNA expression in β-estradiol (E2) treated mIMCD-3 cells after 2 hours of infection with Dr+ *E. coli*

These experiments were conducted to determine the role of E2 in the regulation of TNF-α mRNA expression during Dr+ *E.coli* bacterial infection in mIMCD-3 cells 2 hours post infection (Figure 6). One way ANOVA revealed a significant effects [$F(4,29) = 97.44$, $p < 0.001$]. TNF-α expression in uninfected cells with E2 treatment was not different from uninfected control cells that received vehicle-treatment alone. Dr+ *E.coli* infection in mIMCD-3 cells for 2 hours induced TNF-α mRNA levels that were significantly increased when compared with uninfected untreated control cells as well as uninfected E2 treated cells. Further, Dr+ *E.coli* infected mIMCD-3 cells that received E2 treatment showed enhanced TNF-α mRNA expression. We observed specifically, cytokine mRNA levels were significantly increased when compared with both untreated or E2 treated cells without infection, as well as with untreated Dr+ *E. coli* infected cells (Figure 6). Dr+ *E.coli* infected mIMCD-3 cells that were co-treated with E2 and the ER antagonist ICI 182, 780, showed significant reduction in TNF-α mRNA, and thus, reversal of TNF-α mRNA increase that was observed in infected cells treated with E2 alone. However, TNF-α mRNA levels were comparable in E2 and ICI co-treated cells and in cells with Dr+ *E.coli* infection alone.

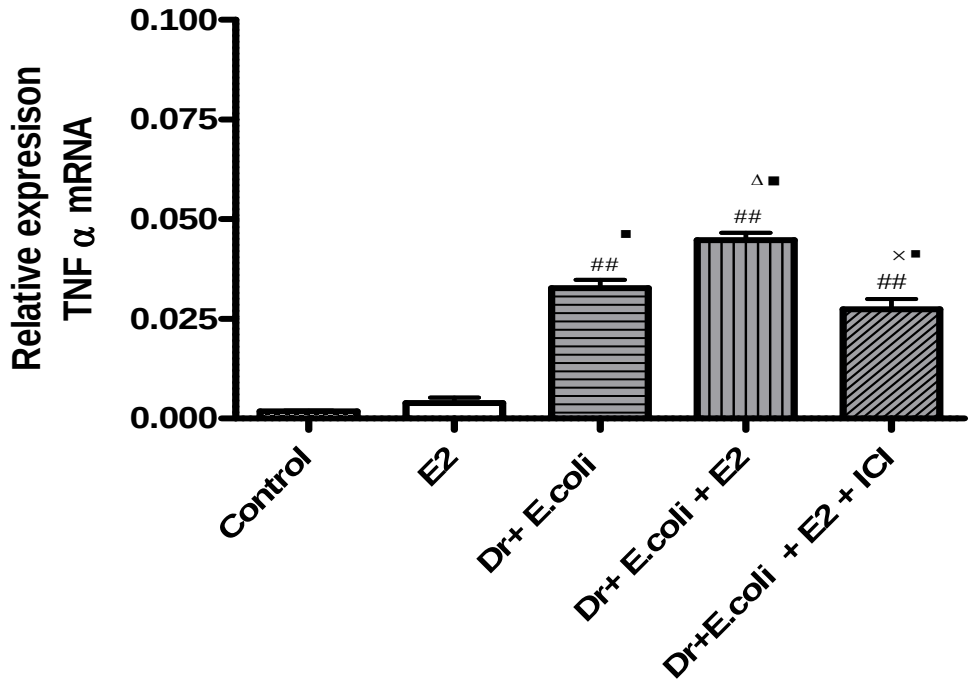

Figure 6: TNF-α mRNA expression in mIMCD-3 cells treated with E2 or with E2 and ICI followed by infection with Dr+ *E. coli* for 2 hours. TNF-α mRNA levels were analyzed by quantitative SYBR green real-time RT-PCR and expressed relative to the housekeeping gene, PPIA. Uninfected and infected mIMCD-3 cells were treated with either 10 nM E2 or co-treated with 10 nM E2 and 10 µM ICI. Untreated cells and 10 nM E2 treated cells without infection served as controls. Error bars are representative of data from 3 experiments, performed in quadruplicate. One-way ANOVA revealed a significant main effect [$F(4,29) = 97.44$, $p < 0.001$]; this was further examined using Tukey's post hoc testing. ##, indicates significant difference relative to untreated control (Control), ■ indicates significant difference relative to E2–treated, uninfected control (E2), Δ indicates significant difference relative to untreated infected cells (Dr+ *E.coli)* × indicates significant difference relative to infected, E2-treated cells (Dr+ *E.coli* + E2).

TNF-α mRNA expression in E2 treated mIMCD-3 cells after 8 hours of infection with Dr+ *E. coli*

These experiments were conducted to determine the role of E2 in the regulation of TNF-α mRNA expression during Dr+ *E.coli* bacterial infection in mIMCD-3 cells at 8 hours post infection (Figure 7). One way ANOVA revealed significant main effects [$F(4,33) = 11.41$, $p < 0.001$]. TNF-α expression in uninfected cells with E2 treatment was not different from uninfected control cells that received vehicle-treatment alone. Dr+ *E.coli* infection in mIMCD-3 cells for 8 hours increased in cytokine mRNA levels; however, TNF-α mRNA was not significantly different when compared with control or E2-treated cells without infection. E2 treatment enhanced TNF-α mRNA expression in infected cells. Specifically, cytokine mRNA levels were significantly increased when compared with control or E2 treated cells without infection, and cells with Dr+ *E. coli* infection alone (Figure 7). Co-treatment with E2 and ICI in infected cells also increased TNF-α mRNA levels when compared to control cells receiving either vehicle treatment or E2 treatment alone, as well as, to those infected with Dr+ *E.coli*. However, levels were not different from Dr+ E.coli infected cells that were treated with E2.

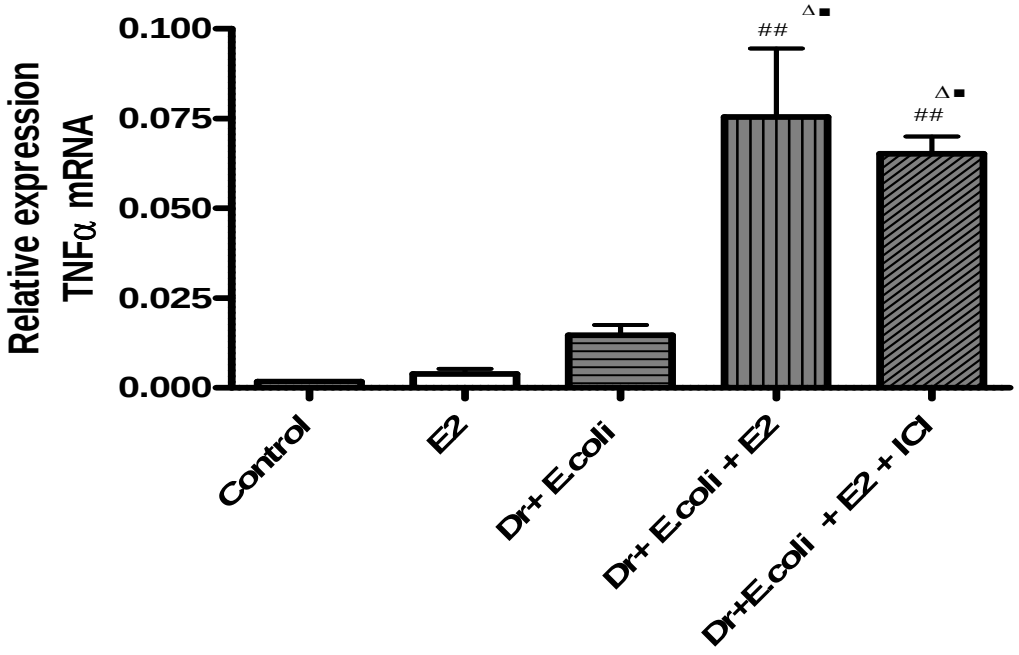

Figure 7: TNF-α mRNA expression in mIMCD-3 cells treated with E2 or with E2 and ICI followed by infection with Dr+ *E. coli* for 8 hours. TNF-α mRNA levels were analyzed by quantitative SYBR green real-time RT-PCR and expressed relative to PPIA. Uninfected and infected mIMCD-3 cells were treated with either 10 nM E2 or co-treated with 10 nM E2 and 10 µM ICI. Untreated cells and 10 nM E2 treated cells without infection served as controls. Error bars are representative of data from 3 experiments, performed in quadruplicate. One-way ANOVA revealed significant main effects [$F(4,33) = 11.41$, $p < 0.001$]. This was further examined using Tukey's post hoc testing. ## indicates significant difference relative to untreated control (Control), ■ indicates significant difference relative to E2–treated, uninfected control (E2), Δ indicates significant difference relative to untreated infected cells (Dr+ *E.coli).*

TNF-α protein expression in E2 treated mIMCD-3 cells after 2 *hours* of infection with Dr+ *E. coli*

These experiments were conducted to determine the role of E2 in the regulation of TNF-α protein expression during Dr+ *E.coli* bacterial infection in mIMCD-3 cells after 2 hours (Figure 8). One way ANOVA revealed significant main effects [$F(4,14) = 8.022$, $p < 0.01$]. TNF-α expression in uninfected cells pretreated with E2 did not differ from uninfected control cells that received vehicle-treatment alone. Dr+ *E.coli* infection in mIMCD-3 cells for 2 hours induced protein levels that were significantly increased when compared with untreated control and with E2-treated control cells without infection. E2 treatment of Dr+ *E.coli* infected cells tended to decrease TNF-α protein levels when compared to *Dr+ E.coli* infected cells without treatment; however, the groups were not statistically different. TNF-α protein levels in E2-treated infected cells were increased when compared with E2-treated cells without infection (Figure 8)

Dr+ *E.coli* infected mIMCD-3 cells that received E2 and ICI co-treatment showed a decrease in TNF-α protein levels when compared with infected cells with or without E2 treatment. Moreover, in TNF-α protein levels were significantly less than those in infected cells.

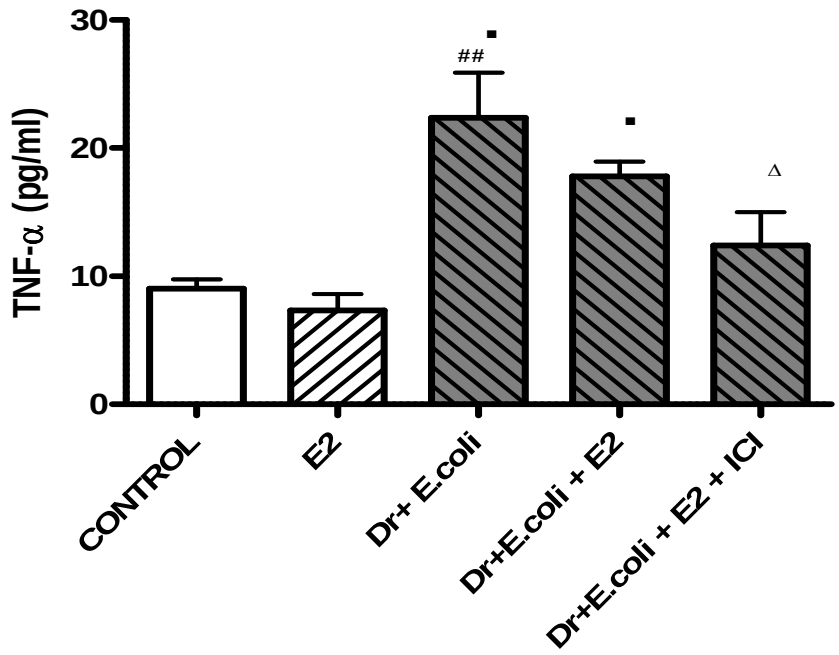

Figure 8: TNF-*α* protein expression in mIMCD-3 cells treated with E2 or with E2 and ICI followed by infection with Dr+ *E. coli* for 2 hours. TNF-α protein levels were analyzed by ELISA. Uninfected and infected mIMCD-3 cells were treated with either 10 nM E2 or co-treated with 10 nM E2and 10 µM ICI. Untreated cells and 10 nM E2-treated cells without infection served as controls. Error bars are representative of data from 3 experiments, performed in quadruplicate. One-way ANOVA revealed significant main effects[$F(4,14) = 8.022$, $p < 0.01$]. This was further examined using Tukey's post hoc testing. ## indicates difference relative to untreated control, ■ indicates significant difference relative to E2-treated, uninfected control ($p<0.05$), Δ indicates significant difference relative to untreated infected cells.

TNF-α protein expression in mIMCD-3 cells after 8 hours infection with Dr+ *E. coli*

These experiments were conducted to determine the role of E2 in the regulation of TNF-α protein expression.

In mIMCD-3 cells infected with *Dr+ E.coli* for 8 hours there were no differences among the various treatment groups (Figure 9). Thus, the levels of TNF-α protein were comparable in all experimental groups at this time point.

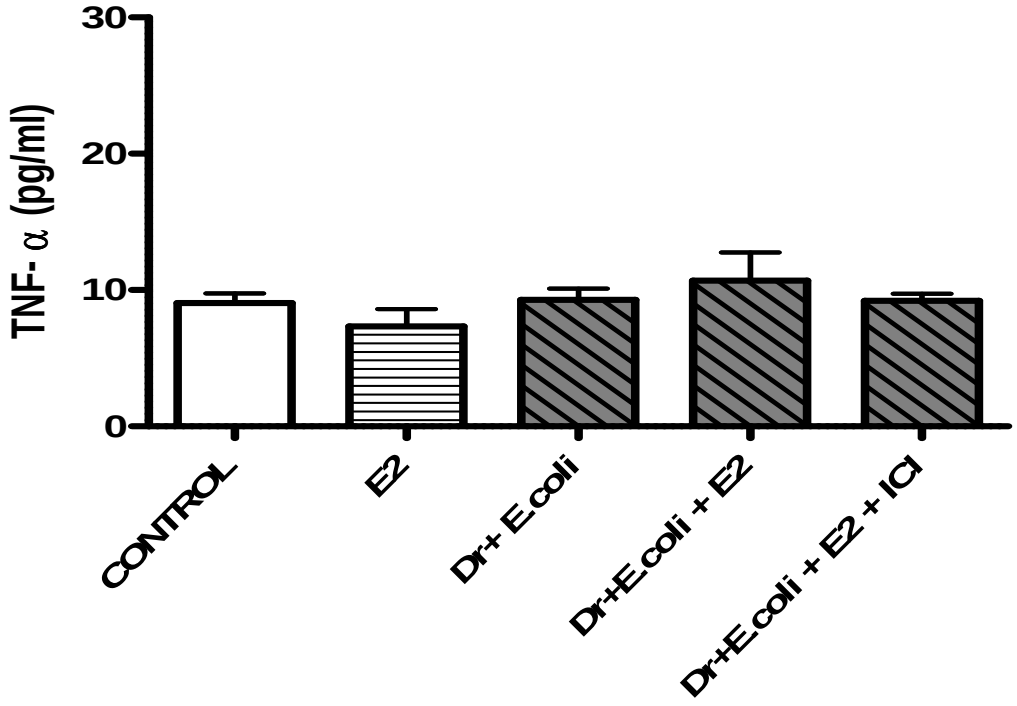

Figure 9: TNF-α protein expression in mIMCD-3 cells treated with E2 or with E2 and ICI followed by infection with Dr+ *E. coli* for 8 hours. TNF-α protein levels were analyzed by ELISA. Uninfected and infected mIMCD-3 cells were treated with either 10 nM E2 or co-treated with 10 nM E2 and 10 µM ICI. Untreated cells and 10 nM E2 treated cells infected with Dr+ *E.coli* served as controls. Error bars are representative of data from 3 experiments, performed in quadruplicate. One-way ANOVA reveled no significant differences overall.

DAF mRNA expression in E2 treated mIMCD-3 cells after 2 hour infection with Dr+ *E. coli.*

These experiments were conducted to determine the role of E2 in the regulation of DAF mRNA expression during Dr+ *E.coli* bacterial infection in mIMCD-3 cells 2 hours after infection (Figure 10). DAF mRNA expression was somewhat variable 2 hours post infection with Dr+ *E.coli*. Infected cells pretreated with E2 appeared to reduced DAF mRNA expression even though there was no significant difference.

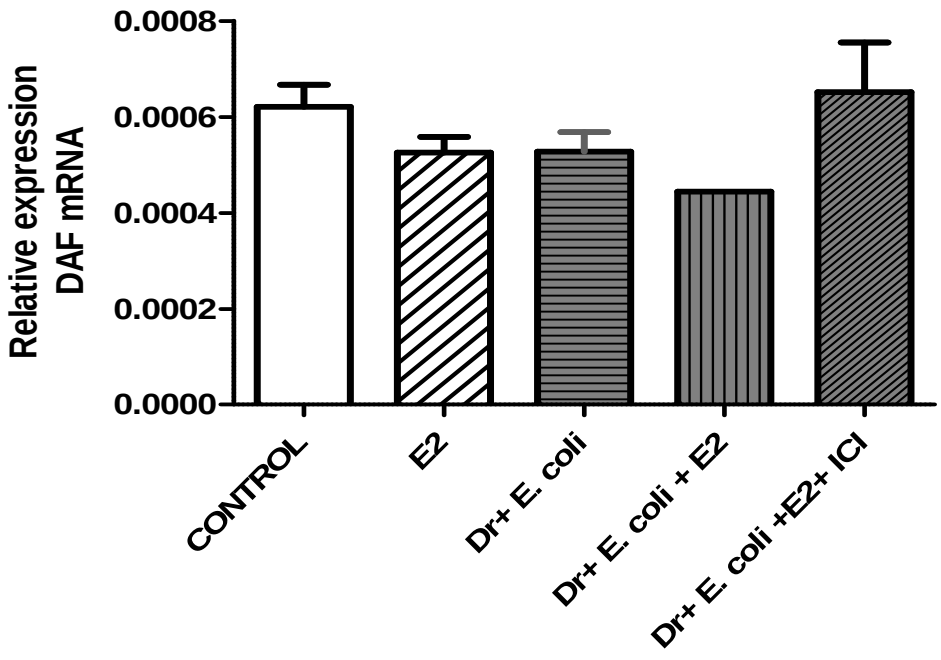

Figure 10: DAF mRNA expression in mIMCD-3 cells treated with E2 or with E2 and ICI followed by infection with Dr+ *E. coli* for 2 hours. DAF mRNA levels were analyzed by quantitative SYBR green real-time RT-PCR and expressed relative to housekeeping gene, PPIA. Uninfected and infected mIMCD-3 cells were treated with either 10 nM E2 or co-treated with 10 nM E2 and 10 µM ICI. Untreated cells and 10 nM E2 treated cells without infection served as controls. Error bars are representative of data from 2 experiments, performed in quadruplicate. One-way ANOVA revealed no significant difference overall.

DAF mRNA expression in E2 treated mIMCD-3 cells after 8 hour infection with Dr+ *E. coli.*

These experiments were conducted to determine the role of E2 in the regulation of DAF mRNA expression during Dr+ *E.coli* bacterial infection in mIMCD-3 cells at 8 hours post infection (Figure 11). One way ANOVA reveled significant main events [F(4,18) = 0. 4954]. DAF expression in infected cells pretreated with E2 showed reduction in DAF when compared to uninfected control cells that received vehicle treatment alone and infected cells co-treated with E2 and ICI but not significantly when compared with untreated infected cells. Thus, E2 and ICI co-treatment significantly reversed the reduction of DAF mRNA levels seen in E2 treated Dr+ *E.coli* infected cells.

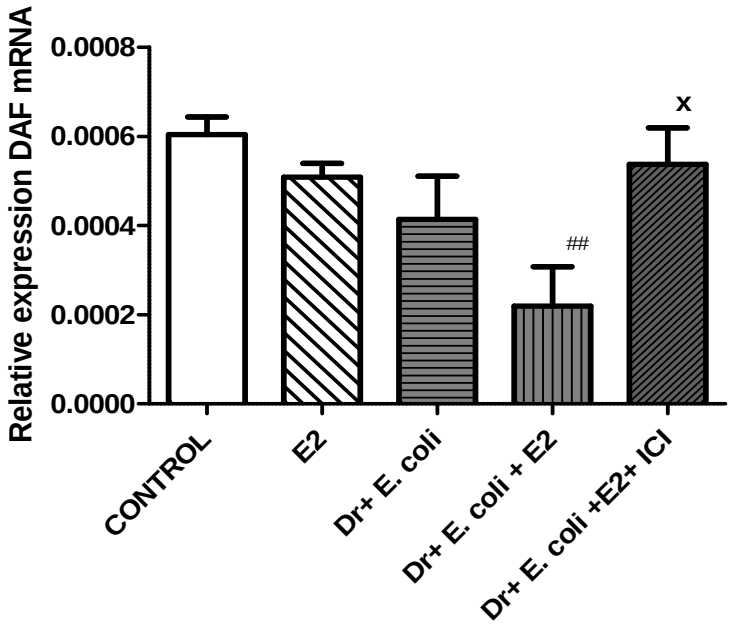

Figure 11: DAF mRNA expression in mIMCD-3 cells treated with E2 or with E2 and ICI followed by infection with Dr+ *E. coli* for 8 hours. DAF mRNA levels were analyzed by quantitative SYBR green real-time RT-PCR and expressed relative to housekeeping gene, PPIA. Uninfected and infected mIMCD-3 cells were treated with either 10 nM E2 or co-treated with 10 nM E2 and 10 µM ICI. Untreated cells and 10 nM E2 treated cells without infection served as controls. Error bars are representative of data from 2 experiments, performed in quadruplicate. One-way ANOVA revealed a significant main effects [F(4,18) = 0. 4954] that were further examined using Tukey's post hoc testing. ## indicates significant difference relative to untreated control (Control), ×: indicates significant difference relative to infected, E2-treated cells (Dr+ *E.coli* + E2).

CHAPTER V

SUMMARY AND CONCLUSIONS

The underlying mechanism of UTI pathogenesis is only partially understood. Despite numerous studies in humans and experimental animals which demonstrate the importance of UPEC virulence factors, host defense mechanisms, and the likelihood of hormonal control, the exact mechanism of UTI pathogenesis remains elusive. From a clinical perspective, the recommended use of estrogen in the prevention of UTI has been only for postmenopausal women who are not receiving oral estrogen and have > 3 recurring UTIs per year (Stamm, 2007). This negates a large population of patients who suffer from UTI. This untreated population, mostly comprised of women, includes pregnant women, of whom 27% have been found to undergo premature labor attributed to inflammation with Dr+ *E.coli* (Kaul *et al.,* 1991; Millar, 1997). This pathological condition, due to its far reaching effects in the population, demands investigation into estradiol's effects during UTI pathogenesis.

In recent studies conducted in an established model of UTI in mIMCD-3 cells, we observed that E2 differentially modulates Dr+ *E.coli* invasion of mIMCD-3 cells in a dose dependent manner (Singh, *et al*, 2010). Specifically, physiological doses of E2 (10nM) significantly decreased bacterial invasion, compared to the cells treated with pharmacological doses of E2 (100nM) or to cells receiving vehicle treatment (Singh *et al*, 2010) .

Additionally, this *in vitro* model of UTI also demonstrated that co-treatment of mIMCD-3 cells with E2 and ICI reversed the E2 protection against of Dr+ *E.coli* invasion in mIMCD-3 cells, suggesting ER involvement.

Based on these findings, the current experiments were conducted in this established *in vitro* model of UTI using 10nm E2 as a means of studying E2 effects on TNF-α production and DAF expression in kidney inner medullary collecting duct cells.

At the onset of infection due to UPEC in the bladder or kidney, epithelial cells stimulate the production of pro-inflammatory cytokines and chemokines such as IL-6, IL-8, IL-1β, and TNF-α, NF-κB and others. Of these cytokines, TNF-α is a key player in mounting a response to infection and is largely implicated in renal inflammation and glomerular damage. TNF-α is known to activate chemokines and adhesion molecules, induce apoptosis, and activate the microbial system of phagocytes (Ernandez, 2009). The E2 mediated regulation of TNF-α has been studied in numerous tissues types and pathologies. It is shown to be up-regulated in both mouse and human kidney infections (Ernandez, 2009; Fahey, 2008).

Under the current experimental conditions, E2 treatment in uninfected mIMCD-3 cells did not give any conclusive data at mRNA or protein levels for TNF-α. This may be attributed to our research design. Uninfected mIMCD-3 cells require additional incubation time points or testing with varying E2 doses to reveal E2 modulation of TNF-α expression. Alternately, absent infection with E2 may not affect TNF-α levels.

In Dr+ *E. coli* infected mIMCD-3 cells with or without E2 treatment, we observed increased TNF-α production at mRNA levels 2 hour after infection. In fact, TNF-α mRNA levels in untreated mIMCD-3 cells infected with Dr+ *E. coli* tended to be increased at both time points. E2 treated, Dr+ *E.coli* infected mIMCD-3 cells, further increased TNF-α mRNA levels. Based on these data, our findings suggest that E2 provides protection against bacterial invasion by up-

regulating TNF-α expression at the message level during the course of infection at 2 hour as well as at 8 hour post infection. Czlonkowska *et al* report that mRNA levels of pro-inflammatory cytokines are regulated by natural changes in estrogen concentration; however, this ability may vary with tissue. Studies by Agace (1993) using an *in vitro* model of UTI in kidney and bladder cells, reported that challenge with *E.coli* bacteria, activates pro-inflammatory cytokine production. Their studies showed that there is a selective cytokine production by epithelial cells following exposure to *E.coli*

To confirm ER involvement in E2 modulation of TNF-α mRNA, mIMCD-3 cells were co-treated with E2 and ICI. E2 co-treatment with ICI inhibited the up-regulation of TNF-α mRNA 2 hours after infection with Dr+ *E.coli*; suggesting that E2-mediated protective effects against Dr+ *E.coli* invasion in mIMCD-3 cells occurs via ER activity. This finding of ER involvement in the protection against invasion agrees with our previous *in vitro* UTI studies (Singh *et al.*, 2010). ICI is a pure ER antagonist and may down-regulate ERα which is the major ER in the kidney. This strongly suggests that E2-mediated modulation of TNF-α in mIMCD-3 cells infected with Dr+ *E.coli* is ER dependent and possibly occurs through ERα (Kuiper *et al 1997)*.

It is unclear why the ICI effect occurred primarily at the 2 hour time point. One possibility is that the continued increase in TNF-α mRNA occurs by a different mechanism that may be independent of ERs. Alternatively, it may be that the ICI concentration we used was insufficient to prevent the E2 effect 8 hours after infection.

Under the experimental conditions of the current study, TNF-α protein levels displayed much variability in infected mIMCD-3 cells, both 2 hours and 8 hours post infection. Dr+ *E. coli* infected cells showed increased TNF-α protein levels only after 2 hours of infection. By 8 hours, TNF-α protein levels were comparable in all treatment groups.

Surprisingly, E2 treatment tended to decrease TNF-α protein levels in cells infected with Dr+ *E.coli* for 2 hours compared to Dr+ *E.coli* infected cells without E2. Additionally, co-treatment of mIMCD-3 cells with E2 and ICI further enhanced the decrease. mIMCD-3 cells infected with Dr+ *E.coli* for 8 hours showed no differences among treatment groups in these experimental conditions, which may suggest that secreted TNF-α protein levels may have peaked just prior to the 8 hour infection time point. However, the fact that TNF-α was elevated 8 hours after infection argues against this interpretation.

The variability seen in TNF-α protein data was seen in 7 replicate experiments, only 3 of which are reported here. This variability in TNF-α protein data may be attributed to a number of experimental or technical factors which require further refinement to allow for firm conclusions. Firstly, consideration of the multiplicity of infection (MOI); that is the ratio infectious agents adjusted to number of seeded cells. Secondly, the sensitivity and loss of receptor function in mIMCD-3 cells sub cultured more than 4 times (note: mIMCD-3 cells were received at passages 15-20) and additional E2 dose and infection time point need further testing. Finally, one important consideration is that the TNF-α protein measured represents secreted protein. Verification using techniques other than ELISA are therefore critical. The examination of protein levels using techniques such as FACS analysis, immunocytochemistry, Western blotting, and confocal microscopy will allow us to assess TNF-α protein localized in the cell.

DAF functions as a major player in host defense against bacterial invasion by regulating the complement system on the membrane. Interaction of UPECs with DAF is the primary method of adherence, invasion, and colonization of kidney epithelium (Selvarangan, 2004). In previous studies, our lab, and others observed that DAF expression is hormonally regulated in uroepithelial cells. (Hasan, 2002; Fang, L. 2004; Selvarangan, 2004, Nowicki *et al*., 1993; Lui, 2004, Mulvey, 2002).

We evaluated infected mIMCD-3 cells at 2 and 8 hour time points to observe the effect of E2 treatment at physiological levels and to ascertain its effects on DAF mRNA in mIMCD-3 cells. Treatment of Dr+ *E.coli* infected mIMCD-3 cells with physiological levels of E2 decreased DAF mRNA expression only after 8 hours. The apparent decrease after 2 hours was not significant suggesting further refinement of experimental parameters may be advantageous. In any case, TNF-α mRNA levels appeared to be inversely regulated in relation to DAF mRNA levels. These findings are consistent with previous studies in which physiological levels of E2 were observed to down-regulate DAF expression in mIMCD-3 cells and to decrease bacterial invasion (Singh *et al*., 2010). To confirm ER involvement in E2 modulation of DAF, mIMCD-3 cells were co-treated with E2 and ICI. Our findings demonstrate that co-treatment of Dr+ *E.coli* infected mIMCD-3 cells with E2 and ICI significantly reversed the decrease in DAF mRNA levels observed at 8 hours post infection, suggesting ER involvement.

In summary, these data suggest that E2-mediated protective effects against Dr+ *E.coli* invasion in mIMCD-3 cells, occurs via up-regulation of TNF-α and simultaneous down-regulation of DAF mRNA. *In vitro* models show that Dr + *E.coli* adhesion factors recognize and adhere to DAF in order to invade and colonize the kidney epithelium which leads to inflammation (Selvarangan *et al*, 2004; Mulvey, 2002). Our studies suggest that E2 treatment elicits protective effects in Dr+ *E.coli* infected mIMCD-3 cells by reducing DAF expression on cells, and thereby reducing bacterial invasion.

As per our hypothesis ,estrogen modulates Dr+*E.coli* invasion in mIMCD3 cells by regulating TNF-α production and affecting expression of DAF. Our current observations of TNF-α mRNA production at 2 and 8 hour time points, as well as DAF mRNA at the 8 hour time point support our hypothesis

Finally, our observations also suggest that E2-affects TNF-α and DAF mRNA expression via ER pathways. This finding is supported by Straub (2007) who suggested that that estrogen levels up-regulate or down-regulate TNF-α expression in a cell type specific manner.

Detailed studies are required, to clarify the modulatory effects of E2 in *Dr+ E. coli* UTI uropathogenesis in kidney IMDCs, and its influence on TNF-α and DAF expression. This would require experiments directly investigating the effects of recombinant TNF-α in mIMCD-3 cells and a further understanding the role of TNF and well as TNF-receptors in E2 modulation of UTIs. Similarly, blocking action of TNF-α by using antibodies in *in vitro* models, or alternatively, using TNF-α knock-out mice will provide further insight into the mechanisms of E2 control of TNF-α during UTIs. The information gained by these studies will lead to the identification of novel biomarkers for the treatment of UTIs.

Further studies are needed to confirm our observations by utilizing additional techniques. These studies will allow us to better understand the mechanism of interaction between host receptor and bacterial ligand, and will aid in the development of novel therapeutics to treat the incidence of UTIs. Recent studies demonstrate implications of TNF-α in UTIs. TNF-α polymorphisms in children with UTIs show renal scarring, demonstrating the effect of TNF-α in the kidney (Savvidou, 2010). Thus, pre-clinical studies are needed to explore the possible benefits of TNF-α therapy in chronic UTIs.

.

REFERENCES

Agace, W., Hedges, S., Andersson, U., Andersson, J., Ceska, M., & Svanborg, C. (1993). Selective cytokine production by epithelial cells following exposure to escherichia coli. *Infection and Immunity, 61*(2), 602-609.

Arenas, I. A., Armstrong, S. J., Xu, Y., & Davidge, S. T. (2005). Chronic tumor necrosis factor-alpha inhibition enhances NO modulation of vascular function in estrogen-deficient rats. *Hypertension, 46*(1), 76-81. doi:10.1161/01.HYP.0000168925.98963.ef

Beato, M., Chalepakis, G., Schauer, M., & Slater, E. P. (1989). DNA regulatory elements for steroid hormones. *Journal of Steroid Biochemistry, 32*(5), 737-747.

Beagley, K. W., & Gockel, C. M. (2003). Regulation of innate and adaptive immunity by the female sex hormones oestradiol and progesterone. *FEMS Immunology and Medical Microbiology, 38*(1), 13-22.

Blango, M. G., & Mulvey, M. A. (2010). Persistence of uropathogenic escherichia coli in the face of multiple antibiotics. *Antimicrobial Agents and Chemotherapy, 54*(5), 1855-1863. doi:10.1128/AAC.00014-10

Calabrese, E. J. (2001). Estrogen and related compounds: Biphasic dose responses. *Critical Reviews in Toxicology, 31*(4-5), 503-515. doi:10.1080/20014091111785

Chassin, C., Goujon, J. M., Darche, S., du Merle, L., Bens, M., Cluzeaud, F., et al. (2006). Renal collecting duct epithelial cells react to pyelonephritis-associated escherichia coli by activating distinct TLR4-dependent and -independent inflammatory pathways. *Journal of Immunology (Baltimore, Md.: 1950), 177*(7), 4773-4784.

Chassin, C., Hornef, M. W., Bens, M., Lotz, M., Goujon, J. M., Vimont, S., et al. (2007). Hormonal control of the renal immune response and antibacterial host defense by arginine vasopressin. *The Journal of Experimental Medicine, 204*(12), 2837-2852. doi:10.1084/jem.20071032

Chen, J., Yang, S., Hu, S., Choudhry, M. A., Bland, K. I., & Chaudry, I. H. (2008). Estrogen prevents intestinal inflammation after trauma-hemorrhage via downregulation of angiotensin II and angiotensin II subtype I receptor. *American Journal of Physiology.Gastrointestinal and Liver Physiology, 295*(5), G1131-7. doi:10.1152/ajpgi.90443.2008

Correale, J., Arias, M., & Gilmore, W. (1998). Steroid hormone regulation of cytokine secretion by proteolipid protein-specific CD4+ T cell clones isolated from multiple sclerosis patients and normal control subjects. *Journal of Immunology (Baltimore, Md.: 1950), 161*(7), 3365-3374.

Curran, E. M., Tassell, A. H., Judy, B. M., Nowicki, B., Montgomery-Rice, V., Estes, D. M., et al. (2007). Estrogen increases menopausal host susceptibility to experimental ascending urinary-tract infection. *The Journal of Infectious Diseases, 195*(5), 680-683. doi:10.1086/511275

Cuzzocrea, S., Genovese, T., Mazzon, E., Esposito, E., Di Paola, R., Muia, C., et al. (2008). Effect of 17beta-estradiol on signal transduction pathways and secondary damage in experimental spinal cord trauma. *Shock (Augusta, Ga.), 29*(3), 362-371. doi:10.1097/shk.0b013e31814545dc

Czlonkowska, A., Ciesielska, A., Gromadzka, G., & Kurkowska-Jastrzebska, I. (2005). Estrogen and cytokines production - the possible cause of gender differences in neurological diseases. *Current Pharmaceutical Design, 11*(8), 1017-1030.

Drekonja, D. M., & Johnson, J. R. (2008). Urinary tract infections. *Primary Care, 35*(2), 345-67, vii. doi:10.1016/j.pop.2008.01.001

Dwyer, P. L., & O'Reilly, M. (2002). Recurrent urinary tract infection in the female. *Current Opinion in Obstetrics & Gynecology, 14*(5), 537-543.

Ernandez, T., & Mayadas, T. N. (2009). Immunoregulatory role of TNFalpha in inflammatory kidney diseases. *Kidney International, 76*(3), 262-276. doi:10.1038/ki.2009.142

Fahey, J. V., Wright, J. A., Shen, L., Smith, J. M., Ghosh, M., Rossoll, R. M., et al. (2008). Estradiol selectively regulates innate immune function by polarized human

uterine epithelial cells in culture. *Mucosal Immunology, 1*(4), 317-325. doi:10.1038/mi.2008.20

Fang, L., Nowicki, B. J., Urvil, P., Goluszko, P., Nowicki, S., Young, S. L., et al. (2004). Epithelial invasion by escherichia coli bearing dr fimbriae is controlled by nitric oxide-regulated expression of CD55. *Infection and Immunity, 72*(5), 2907-2914.

Ferreri, N. R. (2007). Estrogen-TNF interactions and vascular inflammation. *American Journal of Physiology.Heart and Circulatory Physiology, 292*(6), H2566-9. doi:10.1152/ajpheart.00193.2007

Foxman, B. (2003). Epidemiology of urinary tract infections: Incidence, morbidity, and economic costs. *Disease-a-Month : DM, 49*(2), 53-70. doi:10.1067/mda.2003.7

Foxman, B., Zhang, L., Tallman, P., Palin, K., Rode, C., Bloch, C., et al. (1995). Virulence characteristics of escherichia coli causing first urinary tract infection predict risk of second infection. *The Journal of Infectious Diseases, 172*(6), 1536-1541.

Georgiadou, P., & Sbarouni, E. (2009). Effect of hormone replacement therapy on inflammatory biomarkers. *Advances in Clinical Chemistry, 47*, 59-93.

Goluszko, P., Moseley, S. L., Truong, L. D., Kaul, A., Williford, J. R., Selvarangan, R., et al. (1997). Development of experimental model of chronic pyelonephritis with escherichia coli O75:K5:H-bearing dr fimbriae: Mutation in the dra region prevented

tubulointerstitial nephritis. *The Journal of Clinical Investigation, 99*(7), 1662-1672. doi:10.1172/JCI119329

Goluszko, P., Niesel, D., Nowicki, B., Selvarangan, R., Nowicki, S., Hart, A., et al. (2001). Dr operon-associated invasiveness of escherichia coli from pregnant patients with pyelonephritis. *Infection and Immunity, 69*(7), 4678-4680. doi:10.1128/IAI.69.7.4678-4680.2001

Greener, M. (2003, One man's JNK is a scientist's treasure promise found in an apoptotic pathway and its connections to inflammation. *The Scientist, 17*, 32.

Gurgoze, M. K., Akarsu, S., Yilmaz, E., Godekmerdan, A., Akca, Z., Ciftci, I., et al. (2005). Proinflammatory cytokines and procalcitonin in children with acute pyelonephritis. *Pediatric Nephrology (Berlin, Germany), 20*(10), 1445-1448. doi:10.1007/s00467-005-1941-6

Hart, A., Pham, T., Nowicki, S., Whorton, E. B.,Jr, Martens, M. G., Anderson, G. D., et al. (1996). Gestational pyelonephritis--associated escherichia coli isolates represent a nonrandom, closely related population. *American Journal of Obstetrics and Gynecology, 174*(3), 983-989.

Hasan, R. J., Pawelczyk, E., Urvil, P. T., Venkatarajan, M. S., Goluszko, P., Kur, J., et al. (2002). Structure-function analysis of decay-accelerating factor: Identification of residues important for binding of the escherichia coli dr adhesin and complement regulation. *Infection and Immunity, 70*(8), 4485-4493.

Hedlund, M., Duan, R. D., Nilsson, A., Svensson, M., Karpman, D., & Svanborg, C. (2001). Fimbriae, transmembrane signaling, and cell activation. *The Journal of Infectious Diseases, 183 Suppl 1*, S47-50. doi:10.1086/318851

Hopkins, W. J., Gendron-Fitzpatrick, A., Balish, E., & Uehling, D. T. (1998). Time course and host responses to escherichia coli urinary tract infection in genetically distinct mouse strains. *Infection and Immunity, 66*(6), 2798-2802.

Huang, H., He, J., Yuan, Y., Aoyagi, E., Takenaka, H., Itagaki, T., et al. (2008). Opposing effects of estradiol and progesterone on the oxidative stress-induced production of chemokine and proinflammatory cytokines in murine peritoneal macrophages. *The Journal of Medical Investigation : JMI, 55*(1-2), 133-141.

Jahnukainen, T., Chen, M., & Celsi, G. (2005). Mechanisms of renal damage owing to infection. *Pediatric Nephrology (Berlin, Germany), 20*(8), 1043-1053. doi:10.1007/s00467-005-1898-5

Johnson, J. R. (1991). Virulence factors in escherichia coli urinary tract infection. *Clinical Microbiology Reviews, 4*(1), 80-128.

Kamada, M., Irahara, M., Maegawa, M., Ohmoto, Y., Takeji, T., Yasui, T., et al. (2001). Postmenopausal changes in serum cytokine levels and hormone replacement therapy. *American Journal of Obstetrics and Gynecology, 184*(3), 309-314. doi:10.1067/mob.2001.109940

Kaul, A. K., Khan, S., Martens, M. G., Crosson, J. T., Lupo, V. R., & Kaul, R. (1999). Experimental gestational pyelonephritis induces preterm births and low birth weights in C3H/HeJ mice. *Infection and Immunity, 67*(11), 5958-5966.

Kaul, A. K., Kumar, D., Nagamani, M., Goluszko, P., Nowicki, S., & Nowicki, B. J. (1996). Rapid cyclic changes in density and accessibility of endometrial ligands for escherichia coli dr fimbriae. *Infection and Immunity, 64*(2), 611-615.

Kovacs, E. J. (2005). Aging, traumatic injury, and estrogen treatment. *Experimental Gerontology, 40*(7), 549-555. doi:10.1016/j.exger.2005.04.009

Kuiper, G. G., Carlsson, B., Grandien, K., Enmark, E., Haggblad, J., Nilsson, S., et al. (1997). Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology, 138*(3), 863-870.

Kuiper, G. G., Enmark, E., Pelto-Huikko, M., Nilsson, S., & Gustafsson, J. A. (1996). Cloning of a novel receptor expressed in rat prostate and ovary. *Proceedings of the National Academy of Sciences of the United States of America, 93*(12), 5925-5930.

Lambert, K. C., Curran, E. M., Judy, B. M., Lubahn, D. B., & Estes, D. M. (2004). Estrogen receptor-alpha deficiency promotes increased TNF-alpha secretion and bacterial killing by murine macrophages in response to microbial stimuli in vitro. *Journal of Leukocyte Biology, 75*(6), 1166-1172. doi:10.1189/jlb.1103589

Liu, J., Miwa, T., Hilliard, B., Chen, Y., Lambris, J. D., Wells, A. D., et al. (2005). The complement inhibitory protein DAF (CD55) suppresses T cell immunity in vivo. *The Journal of Experimental Medicine, 201*(4), 567-577. doi:10.1084/jem.20040863

Lorenzo, J. (2003). A new hypothesis for how sex steroid hormones regulate bone mass. *The Journal of Clinical Investigation, 111*(11), 1641-1643. doi:10.1172/JCI18812

MacEwan, D. J. (2002). TNF ligands and receptors--a matter of life and death. *British Journal of Pharmacology, 135*(4), 855-875. doi:10.1038/sj.bjp.0704549

Male, D., Brostoff, J., Roth, D., & Roitt, I. (2006). *Immunology* (Seventh ed.). Pennsylvania: mosby Elsevier.

Matthews, J., & Gustafsson, J. A. (2003). Estrogen signaling: A subtle balance between ER alpha and ER beta. *Molecular Interventions, 3*(5), 281-292. doi:10.1124/mi.3.5.281

Metcalfe, P. D., & Meldrum, K. K. (2006). Sex differences and the role of sex steroids in renal injury. *The Journal of Urology, 176*(1), 15-21. doi:10.1016/S0022-5347(06)00490-3

Millar, L. K., & Cox, S. M. (1997). Urinary tract infections complicating pregnancy. *Infectious Disease Clinics of North America, 11*(1), 13-26.

Moggs, J. G., & Orphanides, G. (2001). Estrogen receptors: Orchestrators of pleiotropic cellular responses. *EMBO Reports, 2*(9), 775-781. doi:10.1093/embo-reports/kve185

Mulvey, M. A. (2002). Adhesion and entry of uropathogenic escherichia coli. *Cellular Microbiology, 4*(5), 257-271.

Mulvey, M. A., Schilling, J. D., Martinez, J. J., & Hultgren, S. J. (2000). Bad bugs and beleaguered bladders: Interplay between uropathogenic escherichia coli and innate host defenses. *Proceedings of the National Academy of Sciences of the United States of America, 97*(16), 8829-8835.

Murray, P. R., Rosenthal, K. S., & Pfaller, M., A. (2005). *Medical microbiology fifth edition.* Pennsylvania: Elsevier Mosby.

Nicolle, L. E. (2008). Uncomplicated urinary tract infection in adults including uncomplicated pyelonephritis. *The Urologic Clinics of North America, 35*(1), 1-12, v. doi:10.1016/j.ucl.2007.09.004

Nowicki, B., Hart, A., Coyne, K. E., Lublin, D. M., & Nowicki, S. (1993). Short consensus repeat-3 domain of recombinant decay-accelerating factor is recognized by escherichia coli recombinant dr adhesin in a model of a cell-cell interaction. *The Journal of Experimental Medicine, 178*(6), 2115-2121.

Nowicki, B., Selvarangan, R., & Nowicki, S. (2001). Family of escherichia coli dr adhesins: Decay-accelerating factor receptor recognition and invasiveness. *The Journal of Infectious Diseases, 183 Suppl 1*, S24-7. doi:10.1086/318846

Orlander, J. D., Jick, S. S., Dean, A. D., & Jick, H. (1992). Urinary tract infections and estrogen use in older women. *Journal of the American Geriatrics Society, 40*(8), 817-820.

Paech, K., Webb, P., Kuiper, G. G., Nilsson, S., Gustafsson, J., Kushner, P. J., et al. (1997). Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. *Science (New York, N.Y.), 277*(5331), 1508-1510.

Pham, T. Q., Goluszko, P., Popov, V., Nowicki, S., & Nowicki, B. J. (1997). Molecular cloning and characterization of dr-II, a nonfimbrial adhesin-I-like adhesin isolated from gestational pyelonephritis-associated escherichia coli that binds to decay-accelerating factor. *Infection and Immunity, 65*(10), 4309-4318.

Ramakrishnan, K., & Scheid, D. C. (2005). Diagnosis and management of acute pyelonephritis in adults. *American Family Physician, 71*(5), 933-942.

Rashmi Singh, Senait Assefa, Anil Kaul, and Rashmi Kaul. (2010). Estrogen receptor alpha mediated estrogen protection against dr+ *escherichia coli* invasion in mouse kidney inner medullary collecting duct (mIMCD3) cells. [Abstract]. *194*

Rauchman, M. I., Nigam, S. K., Delpire, E., & Gullans, S. R. (1993). An osmotically tolerant inner medullary collecting duct cell line from an SV40 transgenic mouse. *The American Journal of Physiology, 265*(3 Pt 2), F416-24.

Rudick, C. N., Billips, B. K., Pavlov, V. I., Yaggie, R. E., Schaeffer, A. J., & Klumpp, D. J. (2010). Host-pathogen interactions mediating pain of urinary tract infection. *The Journal of Infectious Diseases, 201*(8), 1240-1249. doi:10.1086/651275

Sakazaki, F., Ueno, H., & Nakamuro, K. (2008). 17beta-estradiol enhances expression of inflammatory cytokines and inducible nitric oxide synthase in mouse contact hypersensitivity. *International Immunopharmacology, 8*(5), 654-660. doi:10.1016/j.intimp.2008.01.007

Savvidou, A., Bitsori, M., Choumerianou, D. M., Karatzi, M., Kalmanti, M., & Galanakis, E. (2010). Polymorphisms of the TNF-alpha and ACE genes, and renal

scarring in infants with urinary tract infection. *The Journal of Urology, 183*(2), 684-687. doi:10.1016/j.juro.2009.10.031

Schaefer, T. M., Fahey, J. V., Wright, J. A., & Wira, C. R. (2005). Innate immunity in the human female reproductive tract: Antiviral response of uterine epithelial cells to the TLR3 agonist poly(I:C). *Journal of Immunology (Baltimore, Md.: 1950), 174*(2), 992-1002.

Selvarangan, R., Goluszko, P., Singhal, J., Carnoy, C., Moseley, S., Hudson, B., et al. (2004). Interaction of dr adhesin with collagen type IV is a critical step in escherichia coli renal persistence. *Infection and Immunity, 72*(8), 4827-4835. doi:10.1128/IAI.72.8.4827-4835.2004

Singh, R. e. a. *Estrogen-mediated modulation of uropathogenic dr+ escherichia coli induced uropathogenesis*. Unpublished Doctor of Philosophy -Biomedical Sciences, Oklahoma State University- Center for Health Sciences, Tulsa,OK.

Sites, C. K., Toth, M. J., Cushman, M., L'Hommedieu, G. D., Tchernof, A., Tracy, R. P., et al. (2002). Menopause-related differences in inflammation markers and their relationship to body fat distribution and insulin-stimulated glucose disposal. *Fertility and Sterility, 77*(1), 128-135.

Sobieszczyk, M. (2008). *Urinary tract infections*. Division of Infectious Diseases Columbia University: Retrieved from http://www.columbia.edu/itc/hs/medical/pathophys/id/2008/utiNotes.pdf

Stamm, W. E. (2007). Estrogens and urinary-tract infection. *The Journal of Infectious Diseases, 195*(5), 623-624. doi:10.1086/511526

Straub, R. H. (2007). The complex role of estrogens in inflammation. *Endocrine Reviews, 28*(5), 521-574. doi:10.1210/er.2007-0001

Styrt, B., & Sugarman, B. (1991). Estrogens and infection. *Reviews of Infectious Diseases, 13*(6), 1139-1150.

Svanborg, C., Frendeus, B., Godaly, G., Hang, L., Hedlund, M., & Wachtler, C. (2001). Toll-like receptor signaling and chemokine receptor expression influence the severity of urinary tract infection. *The Journal of Infectious Diseases, 183 Suppl 1*, S61-5. doi:10.1086/318858

Valles, S. L., Dolz-Gaiton, P., Gambini, J., Borras, C., Lloret, A., Pallardo, F. V., et al. (2010). Estradiol or genistein prevent alzheimer's disease-associated inflammation correlating with an increase PPAR gamma expression in cultured astrocytes. *Brain Research, 1312*, 138-144. doi:10.1016/j.brainres.2009.11.044

Vandewalle, A. (2008). Toll-like receptors and renal bacterial infections. *Chang Gung Medical Journal, 31*(6), 525-537.

Wolfs, T. G., Buurman, W. A., van Schadewijk, A., de Vries, B., Daemen, M. A., Hiemstra, P. S., et al. (2002). In vivo expression of toll-like receptor 2 and 4 by renal epithelial cells: IFN-gamma and TNF-alpha mediated up-regulation during inflammation. *Journal of Immunology (Baltimore, Md.: 1950), 168*(3), 1286-1293.

Wullt, B., Bergsten, G., Connell, H., Rollano, P., Gebretsadik, N., Hull, R., et al. (2000). P fimbriae enhance the early establishment of escherichia coli in the human urinary tract. *Molecular Microbiology, 38*(3), 456-464.

Wullt, B., Bergsten, G., Samuelsson, M., Gebretsadik, N., Hull, R., & Svanborg, C. (2001). The role of P fimbriae for colonization and host response induction in the human urinary tract. *The Journal of Infectious Diseases, 183 Suppl 1*, S43-6. doi:10.1086/318849

Xing, D., Feng, W., Miller, A. P., Weathington, N. M., Chen, Y. F., Novak, L., et al. (2007). Estrogen modulates TNF-alpha-induced inflammatory responses in rat aortic smooth muscle cells through estrogen receptor-beta activation. *American Journal of Physiology.Heart and Circulatory Physiology, 292*(6), H2607-12. doi:10.1152/ajpheart.01107.2006

Zhang, D., Zhang, G., Hayden, M. S., Greenblatt, M. B., Bussey, C., Flavell, R. A., et al. (2004). A toll-like receptor that prevents infection by uropathogenic bacteria. *Science (New York, N.Y.), 303*(5663), 1522-1526. doi:10.1126/science.1094351

.

VITA

Brittany R. Bolt

Candidate for the Degree of

Master of Science

Thesis: ESTROGEN REGULATION OF TUMOR NECROSIS FACTOR ALPHA DURING DR^{+} *ESCHERICHIA COLI* UROPATHOGENESIS

Major Field: Immunology/Microbiology

Biographical:

Personal Data;

Education:
Completed the requirements for the Master of Science in Biomedical Sciences at Oklahoma State University- Center for Health Sciences, Tulsa, Oklahoma in July, 2010

Completed the requirements for the Bachelor of Science in Biology with a minor in Chemistry and Spanish at Missouri State University, Springfield, Missouri, USA 2010.

Name: Brittany Bolt Date of Degree: July, 2010

Institution: Oklahoma State University- Location: Tulsa, Oklahoma

Title of Study: ESTROGEN REGULATION OF TUMOR NECROSIS FACTOR ALPHA DURING DR^{+} ESCHERICHIA COLI UROPATHOGENESIS

Pages in Study: 61 Candidate for the Degree of Master of Science

Major Field: Immunology/Microbiology

Scope and Method of Study:

The purpose of the present study was to investigate the role of estrogen in modulating TNF-α responses during Dr+*E.coli* infection in mIMCD-3 cells. E2 and ER antagonist ICI-182780 pre-treated mIMCD3 cells were infected with Dr+*E.coli* for 2 and 8 hours. TNF-α and DAF expression at mRNA levels were determined by quantitative Real-time RT-PCR and secreted protein quantified by ELISA.

Findings and Conclusions:

E2 modulated TNF-α mRNA levels at both time points in Dr+ *E.coli* infected mIMCD-3 cells. ICI-182, 780 and E2 co-treatment of cells reversed the observed E2 effects on TNF-α mRNA. TNF-α mRNA increase in DR+ *E.coli* infected cells also coincided with down-regulation of DAF mRNA. Significant reversal of DAF mRNA levels was induced by co-treatment of the cells with E2 and ICI. Thus, E2 mediates protective effects against Dr+ *E.coli* invasion in mIMCD3 cells by modulating TNF-α and DAF production.

ADVISER'S APPROVAL: Dr. Rashmi Kaul