

Studies on the Inflammatory Response in Experimental Acute Pyelonephritis and its Importance for the Development of Renal Scarring

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*To my parents, breathers and sisters
Ahmed, Ayat and Nahid*

Abstract

Acute pyelonephritis especially in childhood is a serious bacterial infection, which is common globally. The infecting microorganism is often derived from the patient's own bowel flora and mainly affects the renal parenchyma and pelvis. If left untreated, acute pyelonephritis can lead to renal scarring and renal failure with a need for dialysis and/or renal replacement therapy.

The aim of the present study was to investigate the inflammatory processes involved in acute pyelonephritis and scarring using a murine model system. Thus mice were transurethrally inoculated with *E. coli* (CFT 073) or as control with saline rather than *E. coli*, followed by temporary obstruction.

The mRNA expression of proinflammatory (IL-1, IL-6, IFN- γ and TNF- α) and anti-inflammatory (IL-4, IL-10, IL-12, TGF- β) cytokines were compared in infected and control mice.

These studies showed that an early and sustained up regulation of pro-inflammatory and down-regulating cytokines was observed both locally in the kidneys and systemically in the spleen of infected mice as well as in saline inoculated and obstructed mice, indicating an influence of the obstruction *per se*.

In order to evaluate the effect an angiotensin II type 1 receptor antagonist, losartan, the same experimental model was used. Mice treated with losartan showed a marked reduction of TGF- β mRNA expression and protein secretion compared to saline treated controls. A 50% reduction in cortical scarring was also observed at 3 and 8 weeks compared to control mice, but this difference failed to attain statistical significance ($P = 0.07$).

The importance of IL-6 in the inflammatory response and for bacterial clearance was further studied in a strain of IL-6 deficient mice. Significantly lower TGF- β mRNA and protein levels were found in the kidneys of IL-6 deficient mice compared to their wild type counterparts with acute pyelonephritis. *In vitro* rIL-6 significantly increased TGF- β production from splenocytes in *E. coli* infected IL-6 deficient mice. IL-6 deficient mice displayed more severe histo-pathological indices and retained viable bacteria in their kidneys longer than their wild type counterparts. *E. coli* infection proved to be more lethal in IL-6 deficient mice as well.

High MIP-2 (the mouse equivalent of human IL-8), MCP-1 and RANTES mRNA expression and protein production were found early after bacterial inoculation followed by a gradual decrease in kidney sections and homogenates of mice with acute experimental pyelonephritis.

Stimulation of a human renal epithelial cell line and human primary mesangial cells with the same bacterial antigen led to a rapid increase in IL-8, MCP-1 and RANTES gene expression. Likewise a rapid release of IL-8 and MCP-1 was observed in both cell types, while RANTES release was delayed.

Conclusions: Acute experimental *E. coli* pyelonephritis and obstruction *per se* are both associated with a rapid up-regulation of pro-inflammatory and modulating cytokine and chemokine mRNA expression, both locally and systemically. The angiotensin II type I receptor antagonist, losartan, attenuated TGF- β and reduced cortical scarring. IL-6 is important for bacterial clearance from infected kidneys and also for host survival.

Original papers

This thesis is based on the following papers, which will be referred to by their Roman numerals.

- I. **A. Khalil**, A. Brauner, M. Bakhet, L.G. Burman, G. Jaremko, B. Wretlind, and K. Tullus.
Cytokine Gene Expression during Experimental *Escherichia coli* Pyelonephritis in Mice. *J. Urol.* 1997; 158: 1576-1580
- II. **A. Khalil**, K. Tullus, M. Bakhet, L. G. Burman, G. Jaremko and A. Brauner.
Angiotensin II Type I Receptor Antagonist (Losartan) Down-regulates Transforming Growth Factor- β in Experimental Acute Pyelonephritis. *J. Urol.* 2000; 164: 186-191
- III. **A. Khalil**, K. Tullus, T. Bartfai, M. Bakhet, G. Jaremko and A. Brauner.
Renal Cytokine Responses in Acute *Escherichia coli* Pyelonephritis in IL-6 Deficient Mice. *Clin Exp Immunol.* 2000; 122: 200-206
- IV. O. Hertting, **A Khalil**, G. Jaremko, M. Chromek, M. Bakhet, T. Bartfai, K. Tullus, A. Brauner.
Chemokine Response in Experimental Acute *Escherichia coli* Pyelonephritis. (Manuscript).

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Abbreviations

ACE	Angiotensin converting enzyme
Ang II	Angiotensin II
b.w.	Body weight
CFU	Colony forming unit
CLED	Cystine lactose electrolyte deficient
CRP	C reactive protein
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
HMCs	Human mesangial cells
i.p.	Intraperitoneal
ICAM-1	Intercellular adhesion molecule -1
IFN- γ	Interferon gamma
IL	Interleukin
ISH	<i>In situ</i> hybridization
IVU	Intravenous urography
MCP-1	Monocyte chemoattractant protein-1
MIP-2	Macrophage inflammatory protein-2
MNC	Mononuclear cells
mRNA	Messenger ribonucleic acid
PBS	Phosphate buffered saline
RANTES	Regulated on activation, normal T-cell expressed and secreted
RT-PCR	Reverse transcriptase polymerase chain reaction
TECs	Tubular epithelial cells
TGF- β	Transforming growth factor beta
TNF- α	Tumor necrosis factor alpha
UTI	Urinary tract infection
UUO	Unilateral urethral obstruction
VCAM-1	Vascular cell adhesion molecule-1
VUR	Vesicoureteral reflux

Introduction

Urinary tract infection

Urinary tract infection (UTI) is common, especially in young children and in women. Since not only the clinical picture but also the pathophysiology and the treatment of UTI depends on the region that is infected, it is necessary to localize the infection to the upper or lower urinary tract. Upper UTI (pyelonephritis or febrile UTI) is usually associated with fever of greater than 38.5°C. Lower UTI in which the bladder (cystitis) and urethra (urethritis) are involved is characterized by dysuria, urgency and frequency of micturition with no or low grade fever. Asymptomatic bacteriuria describes the presence of bacteria in the urinary tract without symptoms and is diagnosed by screening. Since infected children often feel better after eradication of the bacteria, the term ‘covert bacteriuria’ might be more appropriate (Tullus and Winberg, 1998).

E. coli is the principle causative pathogen both in uncomplicated upper and lower urinary tract infection. *E. coli* is present in 70 to 85 percent of the cases (Winberg et al., 1974) and is usually derived from the patient’s own bowel flora. A low flow rate together with infrequent and poor bladder emptying predisposes the establishment and multiplication of the bacteria within the urinary tract (Warren, 1996) (Heptinstall, 1998).

Acute pyelonephritis

Acute pyelonephritis is an acute inflammation of the renal parenchyma and pelvis associated with bacterial infection (Winberg et al., 1974) (Jodal, 1987). Pyelonephritis develops when uropathogens colonize the peritoneum, ascend via the urethra into the bladder, and then via the ureters to the kidneys. Clinically it is characterized by sudden onset, fever, malaise, nausea, vomiting, rigors, sweating and flank pain. Elevated C reactive protein, (CRP) or erythrocyte sedimentation rate (ESR) and significant bacteriuria are used to diagnose acute pyelonephritis (Otto et al., 1999). In small children, the dominating clinical picture is fever without localized symptoms (Jodal, 1987) (Tullus and Winberg, 1998).

Vesicoureteral reflux (VUR), obstructive malformations and/or dilated hypotonic ureters may also facilitate the ascent of the infection via the ureters to the kidneys (Warren, 1996). In children with acute pyelonephritis less than 5% were found to

have congenital malformation and 30% VUR (Winberg et al., 1974) (Jodal, 1987). Thus in the majority of children, UTI is due to bacterial virulence factors rather than structural abnormalities. It has been demonstrated that virulent bacteria cause more severe acute symptom (Svanborg Eden and de Man, 1987). UTI can also be a source of life threatening Gram-negative septicemia (Weinstein et al., 1983).

Histopathological changes in the kidneys

The pelvic and calyceal epithelium is affected by the acute inflammatory changes characteristic of acute pyelonephritis. Histopathological examination of this condition in experimental animals reveals that the collecting tubules in the medulla and intertubular capillaries are filled with neutrophils. Extensive tubular destruction occurs followed by rupture of the capillary walls with cellular and fluid leakage into the interstitial space. A few days after the onset of infection, chronic inflammatory cells, e.g. macrophages, lymphocytes and plasma cells appear (Heptinstall, 1998).

Inflammation

Inflammation is a vital consequence of tissue injuries associated with the invasion of pathogens. Inflammatory reactions characterized by redness, hotness, swelling and pain are associated with the accumulation of leukocytes which have migrated to the site of inflammation (Kishimoto, 1992). Cellular immunity is the crucial part of the initiation and/or progression of the kidney diseases (Nikolic-Paterson DJ, 1997). Neutrophils have been identified as local effector cells having antibacterial properties and the ability to clear the bacteria from the urinary tract (Haraoka et al., 1999).

Mucosal inflammatory response

Mucosal surfaces in general are exposed to bacteria that are either transiently or permanently resident in the lumen. In UTI, the infection begins when the pathogen at the mucosal surface contacts the epithelium and triggers inflammation. Bacteria stimulate epithelial cells and other cells in the urinary tract mucosa to secrete pro-inflammatory cytokines and chemokines (Schilling et al., 2001) (Godaly et al., 2000). Both resting and stimulated leukocytes and endothelial cells express multiple adhesion molecules including selectins, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) on their surface. These molecules play a role in the recruitment of leukocytes to the inflamed or infected sites. The pro-

inflammatory cytokines IL-1, TNF- α as well as endotoxin stimulate endothelial cells to express their adhesive molecules. Cellular migration and rolling is primarily mediated by selectins expressed on neutrophils. Adhesion of selectin to its ligand on the endothelium mediates leukocyte rolling in the microvasculature. At areas of inflammation rolling alone the vessel wall permits leukocytes to bind more tightly to activated endothelium. This tight binding allows the leukocytes to extravasate and cross the epithelial barrier (Cronstein, 2000). Leukocytes found in the urine (pyuria) are used as a diagnostic tool of UTI (Tullus and Winberg, 1998).

Renal scarring

Renal scarring (atrophic pyelonephritis or reflux nephropathy), refers to structural damage of the renal parenchyma and is often associated with acute childhood pyelonephritis (Olbing et al., 1992) (Bailey, 1973) (Gordon et al., 1990). Scarring can be unilateral or bilateral and can affect all or a part of the kidney. Renal scars always develop at the site of a previous kidney infection. The diagnosis is based on the gross morphology of the kidney.

Risk factors for renal scarring are congenital dysplasia, obstruction, VUR, young age, delayed treatment and bacterial virulence factors. Congenital renal scarring is the result of dysplasia often associated with prenatal VUR (Dillon and Goonasekera, 1998). Low birthweight is also considered a risk factor for development of renal scarring in childhood (Hellstrom et al., 2001). VUR has been shown to be an important risk factor for the development of renal scarring and occurs in 30-40% of children with UTI (Rushton et al., 1992). High grades of VUR are associated with about 65% renal scarring in children after the first recognized UTI (Jodal, 1987). VUR can also cause repeated episodes of acute infection and produce renal scarring. In some cases, VUR can lead to progressive renal failure. Children with gross reflux (reflux back to the renal pelvis plus ureteral dilatation) are at highest risk. However, new scars can also occur with milder degrees of reflux (Jodal, 1987) (Rushton et al., 1992) and can occur even in the absence of any known risk factor, obvious obstruction or evidence of reflux (Rushton et al., 1992) (Roberts, 1996). Since the papillary-collecting duct orifices are normally wide open at the upper and lower poles in young children, intrarenal reflux usually occurs at these sites. Infants and children

below the age of four years are at higher risk of developing renal scarring as a consequence to UTI (Vernon et al., 1997).

Delayed diagnosis, ineffective antibacterial treatment of pyelonephritis (or no treatment at all) are also risk factors for the development of renal scarring (Tullus and Winberg, 1998) (Roberts, 1999). Even after the infection has been eliminated, existing damage persists and progressive renal fibrosis with a further loss of function can occur in some cases (Sharma et al., 1993).

The diagnosis of renal scarring can be based on:

Intravenous urography, ultrasonography, DMSA scintigraphy, or histopathology. A brief description of these methods in relation to the diagnosis of renal scarring is given in the following sections.

Intravenous urography (IVU):

IVU provides a detailed visualization of the anatomy of renal parenchyma, calyces, pelvis, ureter and bladder (Stokland et al., 1999). Clubbing of calyces overlying parenchymal scarring can be seen. Parenchymal scars appear as an irregular outline or are wedge-shaped with flattening of the renal papillae and a clubbed appearance of the calyces.

Ultrasonography:

Anatomical abnormalities and large scars can be seen with ultrasonography. This method also provides the actual measurement of the kidneys. While ultrasonography is simple and does not require radiation or contrast imaging, it is rather insensitive (Stokland et al., 1999).

DMSA scintigraphy:

Scarring of the kidneys is observed using DMSA (Technetium 99m [^{99m}TC] dimercaptosuccinic acid) in about 40% of children with acute pyelonephritis compared to 10% seen with urography (Webb, 1998) (Stokland et al., 1999). DMSA is taken up by proximal tubular cells such that a localized area of reduced up-take indicates a site of scarring. It is thus a sensitive method for diagnosing renal scarring compared with the IVU. Other advantages over IVU include assessment of differential functions, absence of contrast induced reaction and reduced radiation (Stokland et al., 1999).

Histopathology:

The characteristic feature of renal scarring found at autopsy and in experimental animals is a chronic inflammation that affects the renal interstitium and the tubular part of the nephrons resulting in renal deformation and fibrotic scarring.

Mechanisms in the development of renal scarring

The strong correlation between the degree of interstitial fibrosis and loss of renal function is due to the fact that the tubulointerstitial compartment represents approximately 80% of the total kidney volume. All progressive kidney diseases are the consequence of destructive fibrosis (Eddy, 2000). The inflammatory response within the kidney is essential for tissue damage and eventual scar formation (Johnson, 1997). This process is dependent on the presence of leukocytes in the renal parenchyma (Topley et al., 1989). The progressive renal damage that occurs during acute pyelonephritis is due primarily to the infiltration of leukocytes and the persistence of bacterial antigens within the lesion, rather than to multiplication or presence of the bacteria itself (Glauser, Meylan, and Bille, 1987).

Renal scarring may also result from an acute inflammatory response. The acute response is characterized by activation of circulating leukocytes and renal parenchymal cells which triggers the production of pro-inflammatory cytokines and growth factors. A chronic phase response follows and is characterized by the excessive deposition of collagen and other extracellular matrix (ECM) macromolecules followed by the development of end-stage renal disease (Heptinstall, 1998).

Transforming growth factor beta (TGF- β) is considered the main regulator of the healing process and of fibrosis. When the healing process is completed, TGF- β release is normally interrupted by feedback mechanisms. Persistence of TGF- β production results in the accumulation of ECM proteins and the development of tissue fibrosis (Border and Ruoslahti, 1992). Interstitial myofibroblasts derived from fibroblasts may contribute to interstitial fibrosis through the synthesis and release of α -smooth muscle actin, TGF- β and collagen III. Furthermore, a close relation has been shown between the severity of tubulointerstitial expression of TGF- β and the degree of interstitial fibrosis and renal function impairment (Goumenos et al., 2001).

In children, obstruction is a common cause of end stage renal disease. Renal dysplasia is the result of obstruction early in gestation, while obstructive nephropathy due to late gestation or postnatal obstruction can lead to interstitial fibrosis with irreversible loss of renal function (Sharma et al., 1993). VUR associated with infection is the primary cause of renal scarring in pyelonephritis. Experimentally it has been shown that bladder neck obstruction, increased voiding and resting bladder pressure as well as peristaltic failure of the ureter can lead to transmission of high pressures back to the kidney and result in renal damage (Mendoza and Roberts, 1983).

Experimental pyelonephritis

Pyelonephritis has been established experimentally in different animal models including monkeys, piglets, rabbits, rats and mice. Acute pyelonephritis can be induced as an ascending infection by challenging animals with live bacteria via the transurethral route by inserting a soft polyethylene catheter into the urethral meatus (Glauser, Lyons, and Braude, 1978) (Johnson et al., 1993) (Gupta et al., 1995). Infection can also be created by direct inoculation of the bacteria with a thin needle into the outer cortex of renal parenchyma (Topley et al., 1989) (Matsumoto et al., 1990b) (Hewitson et al., 1998). Obstruction is not only a potent factor initiating infection but also enhances bacterial persistence and multiplication by creating conditions favorable to the bacteria such as a relatively static urine pool. These conditions also favor the spread of infection to the kidneys and facilitate VUR. In experimental models of pyelonephritis, obstruction can be created surgically by ligation of the ureter (UUO) (Sharma et al., 1993) or reversibly by coating the urethral meatus with collodion (Johnson et al., 1993).

***E. coli* virulence factors**

E. coli strains encode several virulence factors that enable them to colonize and persist on the epithelium in spite of a highly effective host defense. Virulence factors are responsible for enhancing the progression of *E. coli* from the fecal reservoir into the bladder and occasionally the kidney. Of greatest importance is the presence of adhesins on the bacterial fimbriae and on the bacterial surface itself. Bacterial adhesion to mucosal or urothelial cells is a general phenomenon determining bacterial virulence. Adhesion of *E. coli* is particularly important when pyelonephritis occurs in an anatomically normal urinary tract. However, it also plays an important role in the

development of cystitis (Svanborg Eden et al., 1988) (Mulvey et al., 2000). Adhesins recognize specific oligosaccharide sequences that are present on the epithelial cell surface as binding sites. The pap adhesin, for example, is present on P fimbriae and recognizes the human digalactoside P blood group on human erythrocytes and urothelial cells where it promotes adhesion (Kallenius, Mollby, and Winberg, 1980) (Kallenius et al., 1982). Type I fimbriae in *E. coli* acts as a virulence factor by promoting persistence and by increasing the inflammatory response to the infection (Roberts, 1991) (Connell et al., 1996) (Martinez et al., 2000). Other factors that affect the uropathogenicity of *E. coli* include the production of hemolysin, which induces formation of pores in the cell membrane (Brauner et al., 1990) (Brauner, Katouli, and Ostenson, 1995) and aerobactin, a siderophore necessary for iron acquisition in the iron-poor environment of the urinary tract (Jacobson et al., 1988; Tullus et al., 1991). Resistance to plasma bactericidal factors is also considered to be a virulence factor (Jacobson et al., 1992).

Cytokines

Cytokines are soluble polypeptides that promote communication between cells. By binding to specific high affinity receptors, cytokines regulate a variety of immune mechanisms. Cytokines are produced in response to different stimuli like bacterial antigens or to cytokines themselves. Various cells produce cytokines, including epithelial cells, endothelial cells and fibroblasts as well as hematopoietic cells (Onishi, Nosaka, and Kitamura, 1998).

Cytokines can act either in an autocrine (on the same cell) or paracrine (on a nearby cell) manner but few of them display an endocrine (on distant cells) action (Fine, Norman, and Ong, 1995). Cytokines released in response to bacterial infection at the mucosal site have local and systemic effects that explain symptoms and signs of the diseases. Systemically in tissues distant from the site of infection, cytokines induce changes in body temperature, blood pressure and blood flow. Locally, inflammation causes recruitment of inflammatory cells, accumulation of toxic products and tissue destruction (Glauser, Meylan, and Bille, 1987).

Cytokine functions are characterized by pleiotropy (single cytokine acts on several different types of target cells and stimulate different responses), redundancy (two or more different cytokines induce similar responses), synergy (combined effect of two cytokines is greater than the sum of individual responses) and antagonism (cytokines

that offset the effect of other cytokines) (Paul, 1989) (Feldmann, Brennan, and Maini, 1996). Cytokines have been classified on several bases depending on their assumed biological actions (Johnson, 1997) (Paludan, 1998). Two major classes of cytokines of particular importance for UTI are the pro-inflammatory and anti-inflammatory cytokines.

Pro-inflammatory cytokines:

These cytokines are involved in the initiation of the inflammatory response to bacterial and viral infections and include IL-1, IL-6, IFN- γ and TNF- α , all of which are important in the early inflammatory cascade. Stimulated macrophages produce IL-1 and TNF- α , which in turn stimulate IL-6 responses. At low concentrations these cytokines act as mediators of local inflammation by activating mononuclear phagocytes and neutrophils and by inducing the expression of adhesion molecules on the epithelial cells. When IL-1, IL-6, and TNF- α are secreted in higher concentrations, they can act in an endocrine manner and induce fever and synthesis of acute phase protein from the liver, as well as septic shock (Lin and Huang, 1998).

IL-1 describes two polypeptides (IL-1 α and IL-1 β) that possess a wide spectrum of inflammatory and immunogenic properties. A variety of cells produce IL-1 in response to infection, microbial toxins and inflammatory agents. Together with IL-6 and TNF- α , IL-1 is considered a pro-inflammatory cytokine with overlapping biological properties. Elevated circulating levels of IL-1 correlate with the severity of infection suggesting that these cytokines participate in the host response to the development of illness. IL-1 and TNF- α share many activities, including the ability to enhance cytokine production and adhesion molecule expression (Feldmann, 2001).

TNF- α is involved in inflammation, fever, septic shock, tumor metastasis, viral replication and autoimmune diseases. Primarily TNF- α is produced by macrophages in response to various inflammatory stimuli including viruses, bacterial and parasite products. TNF- α production is induced by cytokines including IL-1, IFN- γ and TNF- α itself, while IL-4, IL-6, IL-10 and TGF- β inhibit its production (Bharat, 2001).

IL-6 is a multifunctional cytokine with pro-inflammatory and immunoregulatory properties. The main sources of IL-6 are macrophages, endothelial cells and fibroblasts. IL-6 is a stable cytokine and acts as a messenger from the site of infection to systems distant from the locus of infection. When secreted in higher concentrations, IL-6 induces acute phase responses (CRP synthesis) and fever (hypothalamic

temperature regulation). A positive correlation has been demonstrated between local or circulating IL-6 concentration, CRP and fever (Otto et al., 1999).

IFN- γ is produced by granular lymphocytes and activated T cells. IFN- γ is the most potent macrophage activating cytokine, but also activates neutrophils and enhances the microbial killing capacity of phagocytes. IFN- γ stimulates the production of IL-6 in synergy with LPS from monocytes.

Anti-inflammatory cytokines:

IL-4 and IL-10 can inhibit cell-mediated immune responses and thus reduce the tissue damage that occurs during the inflammatory reaction (Paludan, 1998). In addition, TGF- β has anti-inflammatory actions and is a key mediator in the suppression of the immune response.

IL-4 is a pleiotropic cytokine with biological activities on various cell types. IL-4 is produced by activated T cells, mast cells and basophils. Some of its important functions include regulation of B and T cell growth, immunoglobulin expression and an attenuation of monocyte and macrophage inflammatory function. IL-4 and IFN- γ regulate each other's activities through a feedback mechanism (Paludan, 1998).

IL-10 is produced by activated monocytes and T cells in response to several agents including bacterial antigens, IL-4, IL-12 and IL-10 itself. It is involved in the regulation of inflammatory responses and immune reactions and possesses stimulatory activities as well. IL-10 affects the immune system through the modulation of growth factors, cytokines and others mediators. IL-10 suppresses the production of pro-inflammatory cytokines by monocytes and neutrophils.

IL-12 is important in the innate immune response and is considered to be a bridge between the innate and acquired immune systems. IL-12 produced by monocytes and macrophages in response to bacteria, bacterial products and parasites. IL-12 induces IFN- γ and augments its response.

TGF- β is considered the prime fibrosis promoting molecule and thus is a major factor in the pathogenesis of scarring and fibrosis that in extreme cases can lead to organ failure. Five isoforms of TGF- β have been described; three of them existing in mammals. TGF- β 1 has been found in both resident kidney cells and infiltrating leukocytes. TGF- β is a multifunctional cytokine, stimulated by numerous factors including angiotensin II, ischemia, platelet activating factor and endothelin 1 (Border and Noble, 1997) (Border and Noble, 1998). TGF- β can induce its own expression

thus amplifying its biological action (Sterzel, Schulze-Lohoff, and Marx, 1993). TGF- β plays a central role in stimulating the repair of damaged tissue including skin, bone marrow and muscles. Active TGF- β triggers several events that promote fibrosis including formation of interstitial matrix proteins, fibronectin and collagen types I and III. TGF- β also induces the expression of plasmin activator inhibitor 1 and thus delays matrix degradation. Additionally, TGF- β is a potent chemoattractant and stimulates macrophages to release IL-1, TNF- α , platelet-derived growth factor (PDGF), basic fibroblast growth factor (b-FGF) and TGF- β itself (Eddy, 2000) (Strutz et al., 2001). The sum of these properties is the amplification and maintenance of the inflammatory response. On the other hand TGF- β has been shown to down-regulates MCP-1 production (Gerritsma et al., 1998) and at high concentration inhibits IL-8 production (Smith et al., 1996) and mesangial cells proliferation (Jaffer et al., 1989).

Chemokines

Chemokines are chemotactic proteins of low molecular weight that participate in the migration of leukocytes to the site of injured tissue (Segerer, Nelson, and Schlondorff, 2000). Chemokines are secreted in response to exogenous stimuli such as bacteria, LPS and viruses or endogenous stimuli as IL-1, IFN- γ and TNF- α . Chemokines are secreted by a variety of cell types including epithelial cells, endothelial cells and fibroblasts as well as leukocytes (Robertson et al., 1998). They are involved in a variety of immune and inflammatory responses and regulate leukocyte extravasation from the blood and lymph vessels to the site of inflammation (Mahalingam and Karupiah, 1999). *E. coli* stimulates epithelial cells to secrete chemokines (Brauner et al., 2001) and augment chemokine receptor expression, which is used by neutrophils to cross the epithelial barrier (Kabore, Simard, and Bergeron, 1999) (Freudeus et al., 2001).

Depending on the position of the first two of four conserved cysteine residues, chemokines are divided into four subgroups (Schluger and Rom, 1997). CXC chemokines, among them IL-8 or MIP-2 the mouse IL-8 homologue, attract neutrophils and non-hematopoietic cells and are involved in wound healing. CC chemokines such as MCP-1 and RANTES are potent chemoattractants and activators of monocytes and lymphocytes (Egido, 1999). C chemokine (lymphotoxin) recruits lymphocytes. CX₃C chemokine (fractalkine/neurotacin) has been characterized and

recruits T lymphocytes, NK cells and monocytes (Mahalingam and Karupiah, 1999). Since cellular immunity is a crucial component in the initiation and progression of renal diseases, chemokines are of great relevance (Ou and Natori, 1999).

Epithelial, endothelial and mesangial cells as well as fibroblasts secrete IL-8/MIP-2 in response to bacterial challenge, their toxins and to IL-1 β and TNF- α . IL-8 is a potent neutrophil attractant that mediates the recruitment of neutrophils to the site of inflammation. Neutrophil migration across the epithelial barrier is CXCR dependent (Hang et al., 1999) (Frendeus et al., 2000) (Godaly et al., 2000).

MCP-1 is induced by various stimuli from a wide variety of tissues while suppressive cytokines like IL-4 and IL-10 downregulate its expression in many cell types. MCP-1 induces the expression of IL-1 and IL-6 in monocytes. Both human and murine MCP-1 bind only to CCR2 with high affinity. Plasma MCP-1 is elevated in sepsis and its expression has been detected in tissue from many diseases characterized by mononuclear cell infiltration. (Aggarwal, 2001).

RANTES also has chemoattractant properties for monocytes, eosinophils, basophils and T lymphocytes. Its production is stimulated by LPS, IL-1 and TNF- α (Figure 1).

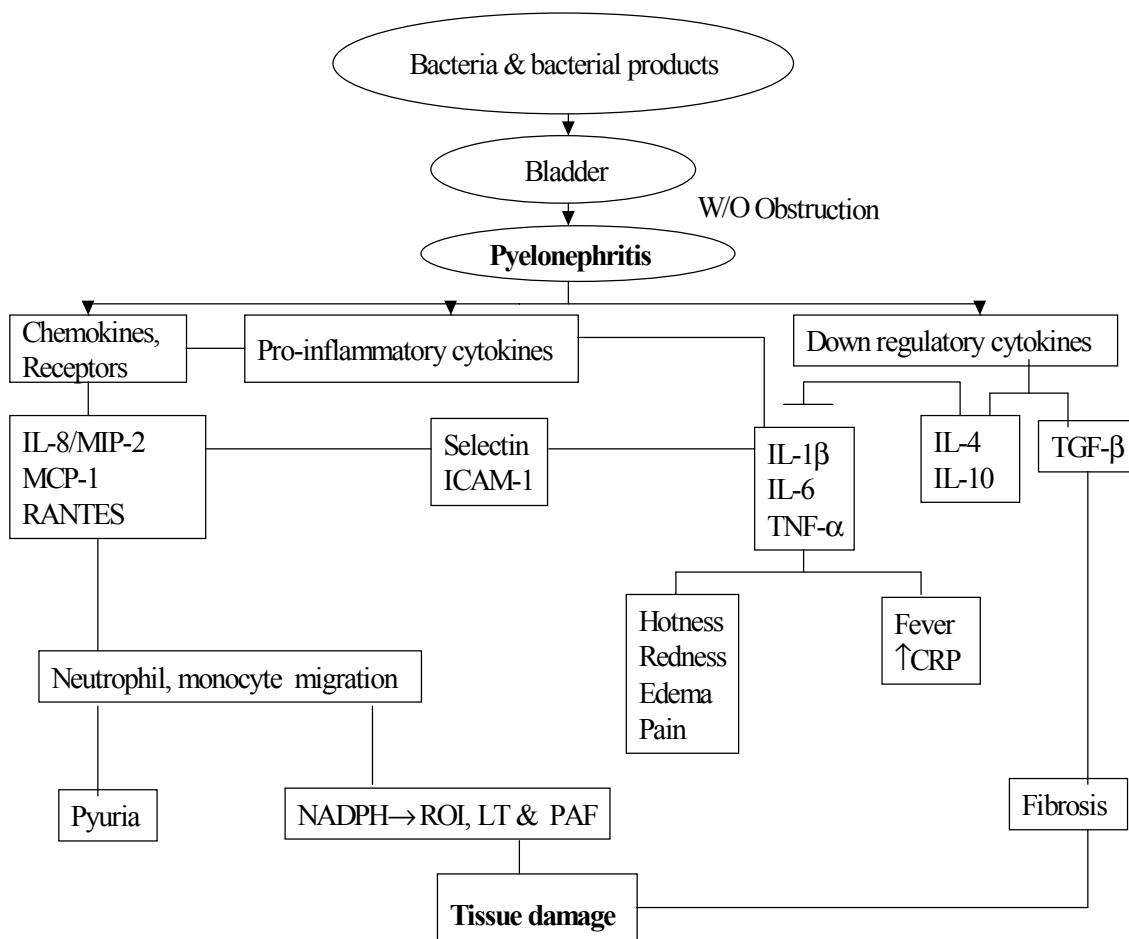


Figure 1: Events leading to tissue damage due to pyelonephritis (W/O) with or without obstruction, (NADPH) nicotinamide adenine dinucleotide phosphate (ROI) radicals oxygen intermediate, (LT) leucotrienes, (PAF) platelet activating factor.

Cytokines and chemokines in UTI

Gram negative UTI (both clinical and experimental) is associated with enhanced production of inflammatory mediators which are mainly produced by resident cells during the early phase and later by mononuclear cells. Pro-inflammatory mediators such as IL-1, IL-6 and TNF- α , chemoattractant cytokines like IL-8 and MCP-1 and anti-inflammatory mediators like IL-10 and TGF- β are all involved in the pathogenesis of UTI.

An early appearance of IL-6 and IL-8 in serum, urine and tissue has been reported in patients and animals with acute pyelonephritis (Jacobson et al., 1994) (Tullus et al., 1994a) (Tullus et al., 1996). Elevated urine concentrations of IL-6 and IL-8 correlate with the concentration of urinary white blood cells (WBC). Hemolysin production by

E. coli increases the magnitude of urinary IL-8 responses (Jantausch, O'Donnell, and Wiedermann, 2000).

Urine and serum levels of MCP-1 are elevated in patients with UTI on admission and decreased after initiation of antibiotic treatment (Olszyna et al., 1999). Interstitial expression of MCP-1 mRNA is present in children with ureteral obstruction and is higher in those with a history of UTI (Bartoli et al., 2000). These findings suggested a potential role of MCP-1 in the pathogenesis of renal damage.

Epithelial IL-1 β , IL-4, IL-6 and IFN- γ production is present in bladder sections from patients with interstitial cystitis and also constitutive production of IL-8 and TGF- β has been observed. The preformed IL-8 is likely to provide a rapid host response in the defense against mucosal microbial attacks (Hang et al., 1998).

Angiotensin II (Ang II)

Juxtaglomerular apparatus synthesize the angiotensin (Ang) precursor, prorenin, which is then cleaved into active renin. Active renin initiates a sequence of steps that begins with cleavage of Ang I from renin substrate (angiotensinogen). Ang I is then converted into the Ang II by an angiotensin converting enzyme (ACE). Ang II has two major systemic effects: systemic vasoconstriction and sodium and water retention. Both of these properties act to reverse the hypovolemia or hypotension that is usually responsible for the stimulation of renin secretion (Rose, 2001).

The actions of Ang II are mediated by the high affinity Ang II-specific receptors, AT₁ and AT₂ that are widely distributed. AT₁ receptors are predominantly found in tissue of adults and mediate Ang II-dependent vasoconstriction. AT₁ receptors are further subdivided into AT_{1A} and AT_{1B}. AT₂ receptors, on the other hand, are expressed in greater abundance in fetal tissues and decline after birth. The actions of the AT₂ receptor are less well understood but may play a role in development (Allen, Zhuo, and Mendelsohn, 1999). Circulating Ang II activates endothelial receptors that are present in all vessels. Tissue receptors are found in many organs such as kidney, heart, brain and lung. Vascular, renal tubular actions and production and degradation of the extracellular matrix are primarily mediated by the AT₁ receptors (Ardaillou, 1999).

Local Ang II production is activated by certain pathological conditions in association with tissue repair including kidneys or blood vessel damage and myocardial infarction

(Allen, Zhuo, and Mendelsohn, 1999). Ang II stimulates TGF- β expression, which is an important mediator of fibrous tissue formation as part of the tissue repair response. Additionally Ang II promotes conversion of latent TGF- β into its biologically active form (Kagami et al., 1994) and stimulates fibroblast collagen synthesis (Sun et al., 2000). It has been suggested that the interaction between the renin-angiotensin system and TGF- β may explain the particular susceptibility of the kidney to fibrosis (Border and Noble, 1998). Ang II stimulates MCP-1 and RANTES expression, where MCP-1 is mediated through AT₁ receptor (Chen et al., 1998) while RANTES expression is mediated through AT₂ receptor (Wolf et al., 1997) (Figure 2). Similarly Ang II stimulates production of nitric oxide (Miyajima et al., 2000a).

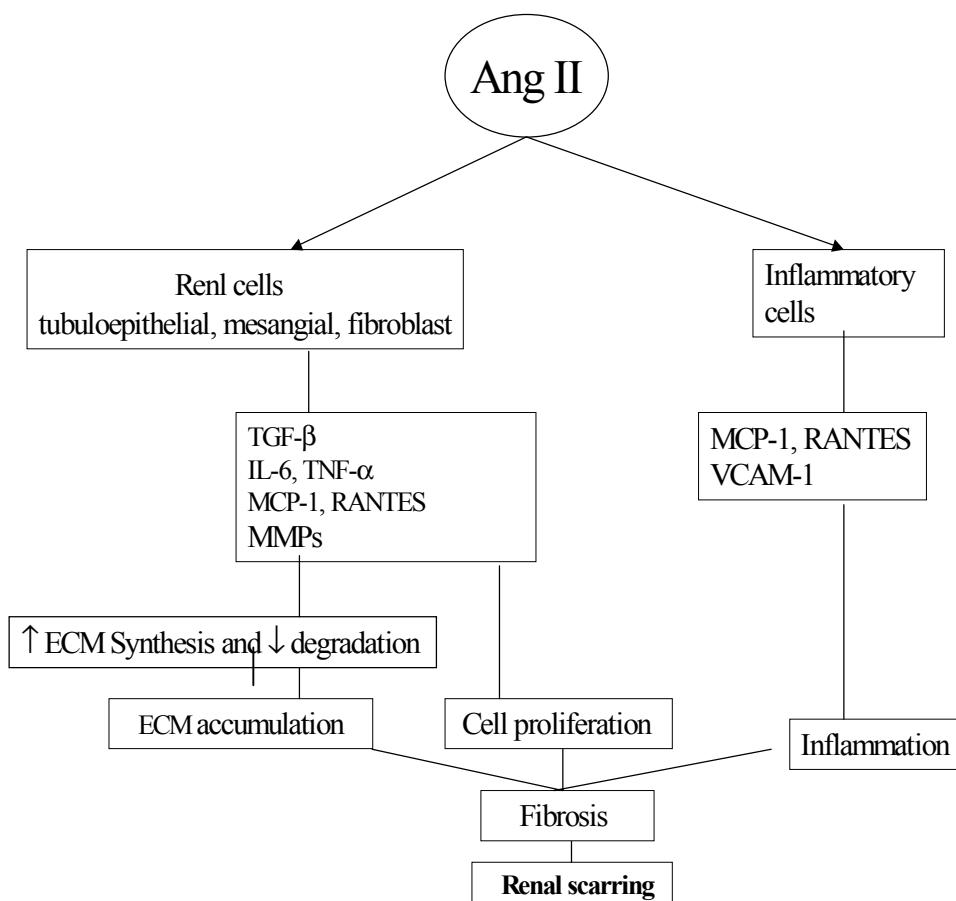


Figure 2: Events leading to tissue damage due to Ang II action. (ECM) Extracellular matrix protein, (MMPs) metalloproteinases, (VCAM-1) vascular cell adhesion molecule-1.

Aims of the study

The general aim of this thesis was to study inflammatory mediators in acute pyelonephritis which can lead to the development of renal scarring.

The specific aims were:

- ◆ To study the cytokine responses in experimentally induced acute pyelonephritis.
- ◆ To investigate the role of the angiotensin II on the production of TGF- β and other cytokines and its influence on renal scarring using the selective type I receptor antagonist, losartan.
- ◆ To investigate the importance of IL-6 in the regulation of inflammation and bacterial clearance in acute pyelonephritis.
- ◆ To study IL-8/MIP-2, MCP-1 and RANTES in experimentally induced acute *E. coli* pyelonephritis and to evaluate the same chemokines after stimulation of human tubular epithelial and mesangial cells with the same antigen and in combination with IL-1 β .

Material and Methods

Animals

Female mice of different strains between the ages of 8 and 10 weeks and weighing from 20 to 30 grams were used for these studies. These strains included:

- I. Bki NMRI outbred mice (Charles River, Uppsala).
- II. IL-6 deficient mice carrying a null mutation in the IL-6 gene and their wild type counterparts (Poli et al., 1994). The generation of these mice is on a B6CBA background.
- III. IL-1 β deficient mice carrying a null mutation in the IL-1 β gene and their wild type counterparts (Zheng et al., 1995). The generation of these mice is on a C57BL/6J background.

Bacteria

E. coli CFT 073 (kindly provided by Dr David E. Johnson, VA Medical center, Baltimore, Maryland, USA) was isolated from a patient with clinical symptoms of acute pyelonephritis. CFT 073 expresses type 1, P and S fimbriae and hemolysin and induces pyelonephritis in experimentally challenged normal mice (Johnson et al., 1993).

Preparation of inoculum

The bacteria were grown overnight on cysteine lactose electrolyte deficient (CLED) agar at 37°C, suspended in PBS and centrifuged for 10 minutes. The cell pellet was thereafter resuspended in PBS to give a final concentration of 10⁹ cfu/ml.

Anesthesia

Inoculation was performed under Hypnorm® (0.1mg/kg fentanyl citrate and 3.3mg/kg fluanisone; 30μl/30g b.w.) and Diazepam® (5 mg/kg, 10μl/30g b.w.) anesthesia. Anesthetics were given i.p. This provided about 3 hours of anesthesia and prevented mice from removing the collodion applied for temporary urethral obstruction.

Inoculation and obstruction

Mice were anesthetized as described above and either infected with *E. coli* or injected with saline. In both cases the inoculum was introduced via a soft sterile polyethylene

catheter (outer diameter 0.61mm and inner diameter 0.28mm, Clay Adams, Becton Dickinson, NJ, USA). 50 µl of either *E. coli* suspension or saline was deposited in the bladder. Immediately after challenge, the catheter was withdrawn. A 6 hour (temporary) obstruction was made by coating the urethral meatus with collodion (Sves 46 Swedish pharmacopia) which was softened and removed with acetone (Johnson et al., 1993). India ink was added to the inoculum to facilitate the detection of urine leakage. The integrity of the collodion seal was documented by the absence of India ink stained urine on the absorbent paper.

Angiotensin II type I receptor antagonist, losartan, treatment

Mice were infected with *E. coli* as previously described and then divided into three groups. Group one and two were treated with losartan. The treatment (daily i.p injections of 40mg or 1mg /kg b.w.) started one day before the bacterial challenge and continued up to 8 weeks. Group three was only injected with saline.

Preparation of tissue sections

At the time of sacrifice, mice were anesthetized by ether inhalation and the abdominal skin was cleaned with alcohol. The skin and the peritoneum were opened through a longitudinal incision. The kidneys and spleen were dissected and removed aseptically. The kidneys were individually weighed, frozen in isopentane on dry ice and stored at -70°C. Sections were cut from the middle of each left and right kidney along the longitudinal axis in 10µm thick sections using a cryotome. Sections were placed on ProbeOn, microscope slides (Fisher, Biotech, Pittsburgh, PA, USA) and stored at -20° C until hybridization experiments were performed. For histopathology, kidney tissue was fixed in 4% buffered paraformaldehyde, embedded in paraffin and 4µm thick sections were collected. In papers III and IV, a new method was used in order to expose most parts of the kidney and to improve the quality of histopathological sections. Each kidney was sliced in a special device with razor blades. Every second slice was mounted with tissue tek in cork and snap frozen in dry ice-cooled isopentane and stored at -70°C. 10µm thick cryotome frozen sections, from both right and left kidney from each mouse were placed on ProbeOn, microscope slides and stored at -20° C until used. The remaining slices were fixed in 4% buffered

paraformaldehyde, embedded in paraffin and cut in 3 μ m thick sections for histopathological assessment.

Preparation of mononuclear cells (MNC)

Mouse spleens were dissected and collected in cold PBS and gently crushed through a stainless steel meshwork. The cells were washed once in tissue culture medium, (Iscove's modified Dulbecco's medium, Flow lab, Irvine, UK). The red blood cells (RBCs) were haemolysed by adding 2ml cold sterile distilled water for 30 seconds, followed by addition of 2ml of 2.7% of saline. The cells were washed twice in tissue culture medium and resuspended to obtain a concentration of 10^6 MNC/ml.

Kidney homogenization

The kidneys used for bacterial culture and ELISA were homogenized in PBS in a glass jar (50mg kidney/ml PBS). The homogenate was centrifuged at 300 \times g for 10 minutes. 10 μ l of the homogenates was added on blood and CLED agar and in a tryptic soy broth. The agar plates were incubated at 37°C for 48 hours. The number of colonies was counted on blood agar. The broth was incubated for 7 days to detect even minute bacterial growth. The supernatants were stored at -70°C for ELISA assay.

Cell culture

A human renal tubular epithelial cell line (HTEC) A498 (ATCC, Rockville, MD, USA) and primary human mesangial cells (HMC) (Clonetics, Walkersville, MD USA) were used in this study.

The HTECs were cultured in cell culture flasks containing Eagles minimal essential medium with 0.1 mM non-essential amino acid, 1.0 mM sodium pyruvate and 10% fetal bovine serum (Life Technologies inc. USA) and incubated at 37°C in 5% CO₂. When cells reached confluence, they were detached from the cell culture flasks with 0.25% trypsin (Sigma Chemical Co, St. Louis, MO, USA), resuspended in medium and seeded into 24-well plates (Nunc, Roskilde, Denmark).

The HMCs express fibronectin but not von Willebrand factor or cytokeratin. The cells were cultured in mesangial cell basal medium (Clonetics) supplemented with 5% fetal bovine serum and 50 μ g/ml of gentamicin and amphotericin (Clonetics). Cells were

grown to confluence and then detached with 0.25% trypsin/EDTA (Clonetics) and seeded onto 24-well plates.

Cell stimulation

Confluent layers of HTECs and HMCs were washed once with PBS in order to remove any non-adhering dead cells and serum-free medium containing *E. coli* CFT 073 (10^8 cfu/ml), IL-1 β (10 ng/ml) or both was added. Supernatants were collected at 0h, 2h, 6h and 24h, centrifuged at 300 \times g for 10 min and stored in -20°C until ELISA analysis was performed.

***In situ* hybridization (ISH)**

ISH involves the covalent bonding of labeled single stranded fragments of DNA or RNA to complementary sequences of cellular DNA or RNA under conditions that promote the formation of stable hybrids. ISH was performed as described for tissue sections (Dagerlind et al., 1992) and modified for cell suspensions (Zhu et al., 1994). Synthetic oligonucleotide probes were labeled using ^{35}S deoxyadenosine-5'-a-(thio)-triphosphate (New England Nuclear Cambridge, MA, USA) with terminal deoxynucleotidyl transferase (Amersham, little Chalfont, UK). A mixture of two to four different approximately 30-48 base pair oligonucleotide probes was used to increase the sensitivity of the method. The oligonucleotide sequences were obtained from GenBank (Scandinavian Gene Synthesis AB, Köping, Sweden) and probes were designed using Mac Vector software. As control probes, the sense sequence for cytokine and chemokine was used in parallel on cells or tissue sections from each animal. These sense control probes failed to label any cells. Omission of fixation resulted in both stronger specific labeling and background reduction as described previously (Dagerlind et al, 1992). Fixation with formalin increased the nonspecific labeling of probes. Emulsion autoradiography was performed and cells expressing more than 15 grains in their cytoplasm were counted by dark field microscopy at 100 \times magnification. Accuracy was checked at higher magnification with bright field illumination. Results were expressed as the number of labeled cells per 100 mm 2 tissue section or 10^5 spleen cells. The tissue section areas were measured by image analysis (Seescan-Image Analysis System, Cambridge, UK), or by graded mesh lens counter.

Enzyme-linked immunosorbent assay (ELISA)

The ELISA technique employed was based on the quantitative sandwich method using monoclonal antibodies. This method measures biologically active TGF- β , MCP-1, MIP-2 and RANTES in supernatants from homogenized kidneys or spleen cells. Kits were obtained from R&D systems (Abingdon, Oxon, UK). The detection limits were 31.2, 2.0, 1.5 and 2.0 pg/ml respectively. Supernatants from stimulated HTECs and HMCs were used to determined IL-8, MCP-1 and RANTES. Kits were obtained from R&D systems The limits of detection were 10.0, 5.0 and 8.0 pg/ml respectively.

Extraction of RNA for PCR

Total RNA extraction from stimulated HTECs and HMCs was performed by the guanidine/phenol method using RNazolTM B, (Biotecx Laboratories, Houston, TX, USA). The cells were lysed directly in the 24-well plates by the addition of 200 μ l of RNazolTM B per well and by passing the lysate through the pipette according to manufacture's instruction. Chlorophorm 200 μ l per 2ml of homogenate was added, mixed and centrifuged at 12,000 $\times g$ for 15min, 4°C. The homogenate was separated into 2 phases, the upper aqueous phase containing RNA was transferred to another tube. RNA was washed by vortexing with 75% ethanol and centrifuged for 8 minutes at 7,500 $\times g$ at 4°C. The final RNA pellet was air-dried and resuspended in 10 μ l of diethyl-pyrocarbonate-treated water. 2 μ l of the solution was used for spectrophotometric determination of the RNA concentration and quality (230-320nm profile).

Reverse transcriptase polymerase chain reaction (RT-PCR)

RT-PCR was used to detect the levels of the chemokines IL-8, MCP-1 and RANTES mRNA in HTECs and HMCs.

One μ g RNA was reverse transcribed to cDNA prior to PCR amplification using random hexamer primers pd(N)₆ (Amersham Pharmacia Biotech, Uppsala, Sweden). A mixture of 5 \times buffer, DTT, dNTP and RNAsin. SuperScript RNase H⁻ (GibcoBRL, Life Technologies) was added and the mixture was incubated at 45°C for 2h and then stored at -60°C.

The chemokines (human IL-8, MCP-1 and RANTES) primers were obtained from Innovagen (Lund, Sweden). The primer for the housekeeping gene, human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was obtained from CyberGene AB, (Huddinge, Sweden).

Amplification was performed with Taq polymerase (GibcoBRL, Life Technologies) in a DNA Thermocycler 480 (Perkin Elmer, Norwalk, CT, USA). All PCR mixtures contained a final concentration of 2 mM MgCl₂, 0.2 mM dNTP, PCR buffer and 0.5μM for each primer.

After 1 min at 95°C, PCR was conducted for a total of 26 (MCP-1), 28 (IL-8), 30 (RANTES) and 28 (G3PDH) cycles under the following conditions: 1 min of denaturation at 94°C, 1 min of annealing at 62, 62, 61 and 60°C, respectively, and 1 min of extension at 72°C, followed by a final extension for 5 min at 72°C and cooling to 4°C.

The PCR products were separated by electrophoresis on a 1.5% agarose gel (GibcoBRL, Life Technologies). The gels were stained with ethidium bromide and photographed. The intensity of the bands was measured under UV-light using Gel Doc 2000 (Bio Rad, USA) and the product/G3PDH intensity ratios were calculated.

Results

Early and sustained up regulation of cytokine mRNA both locally in the kidneys and systemically in the spleen (paper I):

The mRNA expression of IL-1 β , IL-4, IL-6, IL-10, IL-12, IFN- γ , TNF- α , TNF- β and TGF- β locally in the kidneys and systemically in the spleens of mice with acute experimental pyelonephritis and the effect of the obstruction *per se* was investigated at 12 hours, 48 hours and 6 days.

Cytokine response in the kidneys:

In kidneys, a significant increase in the number of cells expressing mRNA of the pro-inflammatory cytokines IL-1 β (Fig. 1) and TNF- α was found at 12 hours compared to unmanipulated animals and compared to obstructed non-infected mice. IL-6 and TNF- β mRNA were increased significantly after 48 hours and 6 days in the infected animals both compared to obstructed, non-infected and to unmanipulated animals (time point 0h) respectively. In the infected mice, TGF- β (Fig. 2) and IL-4 mRNA expression were significantly elevated at 48 hours compared to obstructed non-infected mice, while IL-10 was significantly increased at 6 days and compared to unmanipulated animals

The non-infected obstructed kidneys showed an increase in the number of cells expressing IL-1 β , IL-4, IL-6, IL-10, IFN- γ , TNF- α and TGF- β mRNA compared to those of unmanipulated animals but at lower levels than the infected kidneys. These increases appeared at 12 hours and persisted for up to 6 days, indicating that the 6 hours obstruction *per se* can influence cytokine expression in the kidneys.

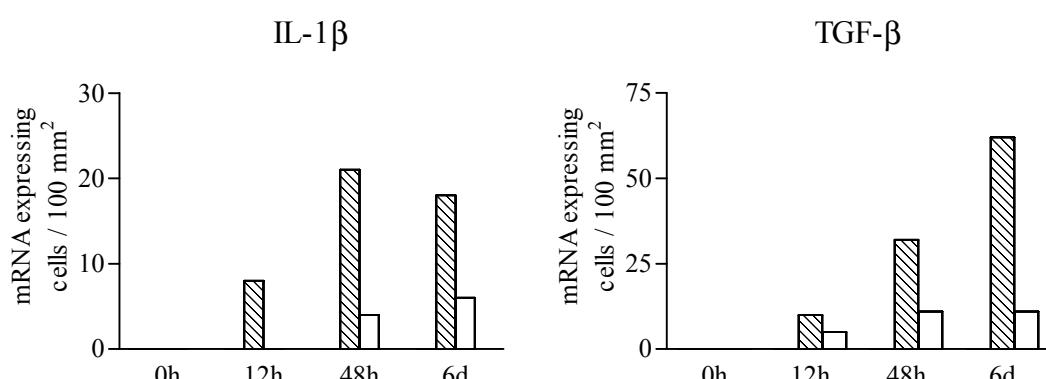


Figure 1 and 2: IL-1 β and TGF- β mRNA expression in kidney sections. Hatched bars represent *E. coli* infected mice, unfilled bars represent obstructed non-infected mice.

Systemic cytokine responses:

Splenocytes from infected mice expressed significantly higher mRNA levels for IL-1 β (Fig. 3), IL-4, IL-10, IL-12, IFN- γ , TNF- α and TGF- β (Fig. 4) at all time points compared to unmanipulated mice. IL-6 mRNA expression was higher at 12 hours and 6 days. Together this indicates a systemic cytokine response as well. The non-infected obstructed mice displayed higher IL-1 β at all time points. TNF- α was elevated at 12 hours and 48 hours compared to unmanipulated mice.

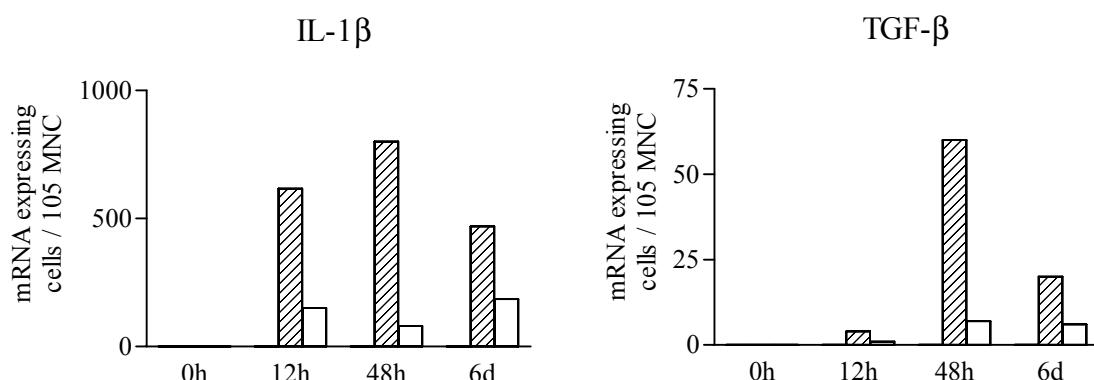


Figure 3 and 4. IL-1 β and TGF- β mRNA expression in spleen MNC. Hatched bars represent *E. coli* infected mice, unfilled bars represent obstructed non-infected mice.

Losartan, treatment markedly reduced TGF- β mRNA expression and protein production (paper II):

The role of the angiotensin II in mediating the effects of acute experimental pyelonephritis on the renal expression of TGF- β , IL-1 β , IL-4, IL-6, IL-10, IL-12, IFN- γ and TNF- α mRNA and on TGF- β protein production was investigated using the angiotensin II type I receptor antagonist, losartan. Bacterial clearance, renal growth retardation and cortical scarring were also studied at 48 hours, 3 and 8 weeks post inoculation.

Losartan, treatment down-regulated TGF- β , IL-6, and IFN- γ :

The number of cells expressing TGF- β mRNA gradually decreased over time in groups treated with 1mg or 40 mg/kg of losartan and were significantly lower than controls at 3 and 8 weeks. Levels of TGF- β mRNA expression did not change in controls, over the eight week study period (Fig. 5). Mice treated with 40 mg/kg b.w of losartan showed significantly lower TGF- β production in the supernatants compared to saline treated mice at 48 hours ($P < 0.005$). IL-6 (Fig. 6) and IFN- γ mRNA

expressions were also significantly lower in the losartan treated groups compared to controls.

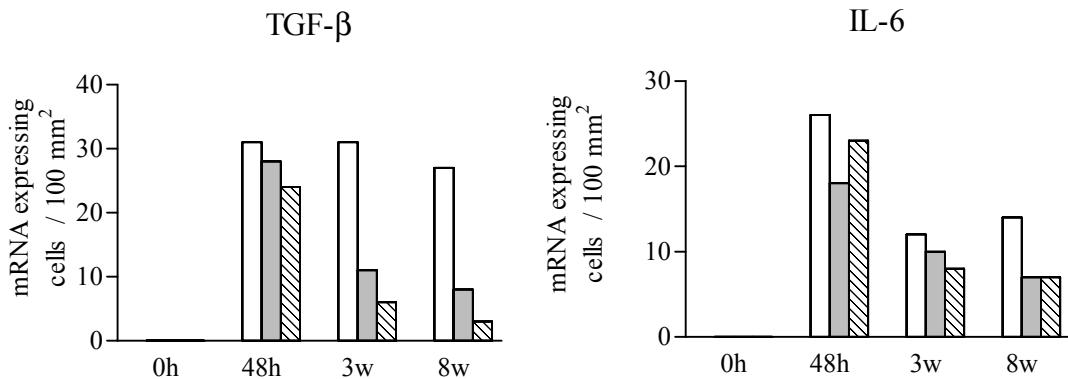


Figure 5 and 6. TGF-β and IL-6 mRNA expression in kidney sections. Open bars represent control mice, filled bars mice treated with 1mg losartan and hatched bars mice treated with 40mg losartan.

Bacterial clearance and losartan treatment:

Viable bacteria were recovered from all mice (8/8) in both the losartan treated and control groups at 48 hours. At 3 weeks, 8/8 of the losartan treated group and 5/8 of the control group contained viable bacteria. The mean renal bacterial count was higher in the losartan treated group compared to the controls at 3 weeks ($P < 0.006$). At 8 weeks, no viable bacteria were found in either group.

Infection-induced kidney weight reductions were not prevented by losartan treatment. Although on average, losartan appeared to reduce the spread of renal scarring by 50%, the reduction was not statistically significant.

IL-6 influences TGF-β expression, enhances bacterial clearance and improves host survival (paper III)

The role of IL-6 in experimental acute *E. coli* pyelonephritis in relation to cytokine mRNA expression, bacterial retention, renal scarring and mortality was studied at 48 hours, 6 days and 3 weeks post inoculation in IL-6 deficient mice and their wild type controls.

Influence of IL-6 on the cytokine response:

In IL-6 deficient mice, TGF-β mRNA expression declined significantly at 48 hours both in *E. coli* infected mice and in saline inoculated compared to the wild type mice (Figure 7 and 8).

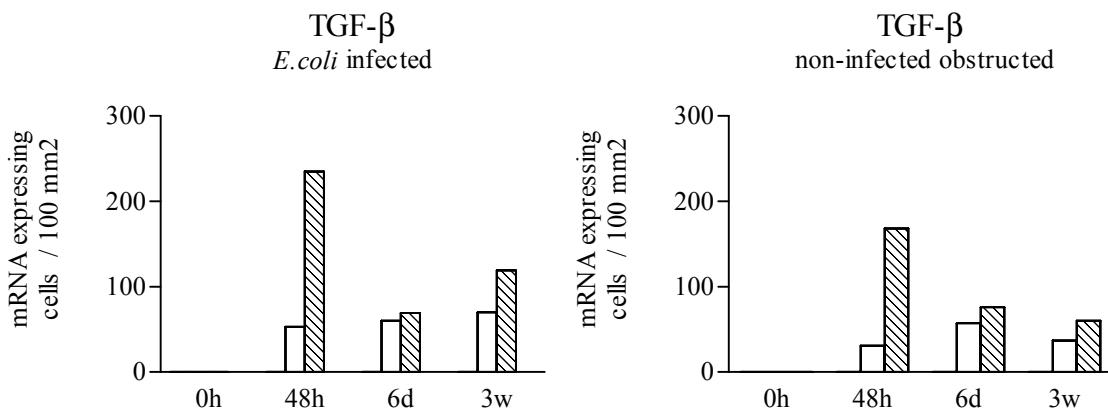


Figure 7 and 8. TGF-β mRNA expression in kidney sections from *E. coli* infected and saline injected mice, open bars represent IL-6 deficient mice, hatched bars represent wild type mice.

At the same time point, the protein content in homogenized kidney supernatants from the infected IL-6 deficient mice also decreased significantly compared to the wild type mice (Figure 9).

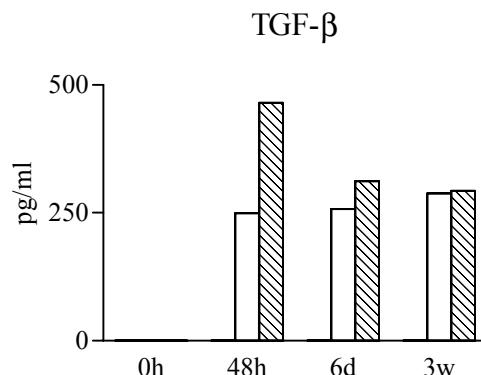


Figure 9: TGF-β production in kidney supernatants from *E. coli* infected mice, open bars represent IL-6 deficient mice, hatched bars represent wild type mice.

The mortality rate of IL-6 deficient mice was greater than wild type controls after *E. coli* infection. Eighteen percent of the IL-6 deficient mice died compared to 6% of the wild type. IL-6 deficient mice also retained viable bacteria in their kidneys longer than their wild type counterparts (Table 1).

	IL-6 deficient	Wild type
48 hours	4/5	3/4
6 days	4/5	0/5
8 weeks	1/4	0/4

Table 1: Number of mice with kidney bacterial growth from the total number of infected mice.

Histopathological changes were observed more frequently in IL-6 deficient than in the wild type mice (Table 2).

	IL-6 deficient	Wild type
48 hours	9/16	7/15
6 days	4/5	2/4
8 weeks	2/4	0/4

Table 2: Number of mice with kidney inflammation from the total number of the infected mice.

Renal chemokine mRNA expression and protein production (Paper IV)

MIP-2, MCP-1 and RANTES mRNA expression and protein levels were investigated in kidney sections from mice infected with *E. coli* or injected with saline and obstructed for 6 hours. HTECs and HMCs were stimulated *in vitro* with *E. coli*, IL-1 β or both and mRNA and protein levels of IL-8, MCP-1 and RANTES were studied.

Chemokine expression and induction:

High levels of MIP-2, MCP-1 and RANTES mRNA expression were found 24 hours post inoculation in both the infected and saline injected groups. Protein levels and mRNA expression for each of these chemokines declined gradually thereafter. MIP-2 mRNA expression and protein production were significantly higher in the *E. coli* infected mice compared to the saline injected controls at each time point (Fig10 and 11).

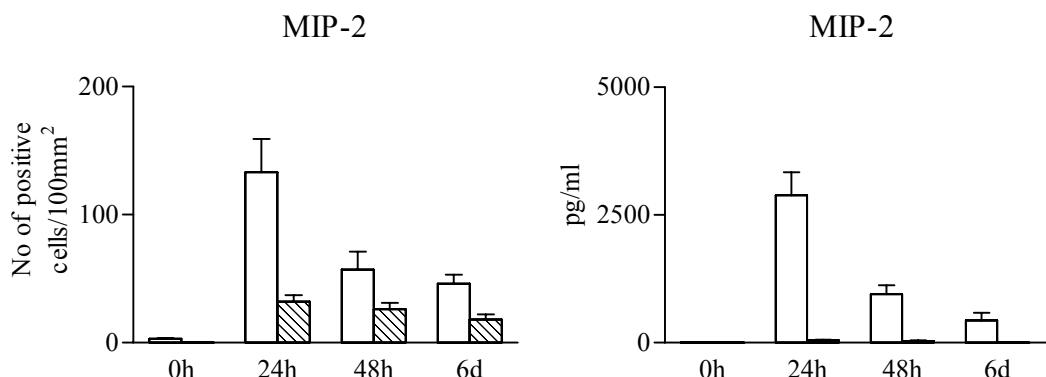


Figure 10 and 11. MIP-2 mRNA expression and production. Open bars represent *E. coli* infected mice, hatched bars represent saline injected mice.

MCP-1 mRNA expression tended to be higher in the kidneys of *E. coli* infected mice compared to saline injected mice, but this difference failed to attain statistical significance (Fig 12). MCP-1 protein levels, however, were significantly higher at 24 hours and 48 hours in the *E. coli* infected compared to saline injected mice (Fig 13).

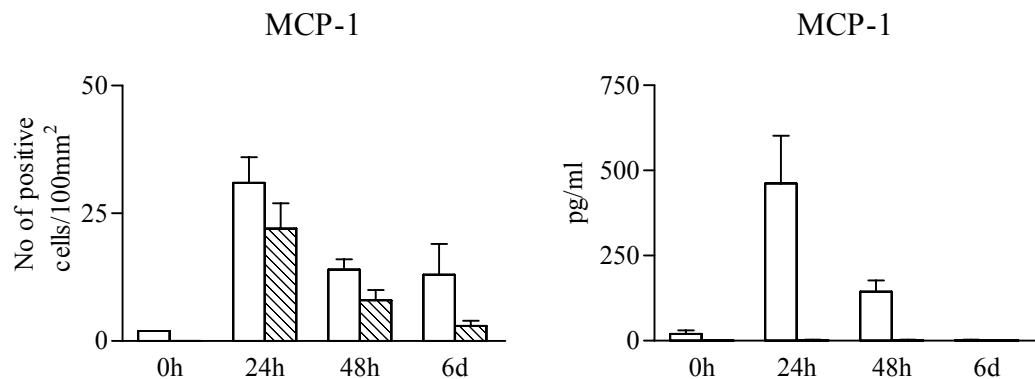


Figure 12 and 13. MCP-1 mRNA expression and production. Open bars represent *E. coli* infected mice, hatched bars represent saline injected mice.

Both RANTES mRNA expression and protein levels were markedly enhanced in *E. coli* infected mice at 24 and 48 hours compared to the saline injected controls (Fig 14). RANTES protein levels remained higher also at 6 days (Fig 15).

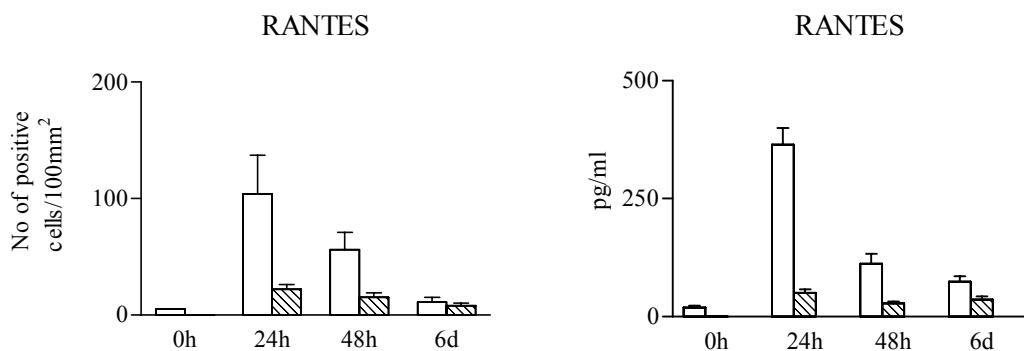


Figure 14 and 15. RANTES mRNA expression and production. Open bars represent *E. coli* infected mice, hatched bars represent saline injected mice.

Chemokine concentration in cell supernatants:

IL-8 protein was increased in both HTECs and HMCs in response to *E. coli* or IL-1 β at 6 hours and further increased at 24 hours. Combined stimulation increased IL-8 production over that of either *E. coli* or IL-1 β alone ($P < 0.001$) (Fig 16 and 17).

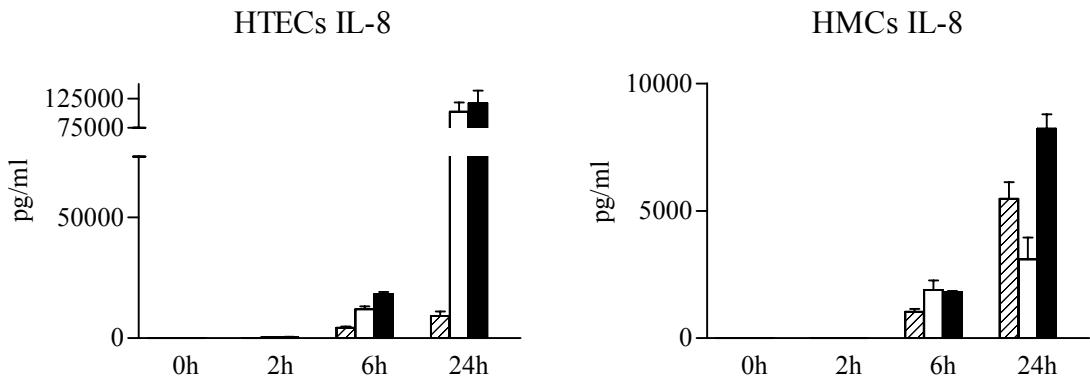


Figure 16 and 17. IL-8 production in the supernatants from HTECs and HMCs. Hatched bars represent *E. coli* infected, open bars cells incubated with IL-1 β and filled combined stimulation (*E. coli* and IL-1 β).

Both *E. coli* and IL-1 β stimulated MCP-1 synthesis in HTECs as well as in HMCs. Interestingly the combination of *E. coli* and IL-1 β decreased the MCP-1 levels (Fig 18 and 19).

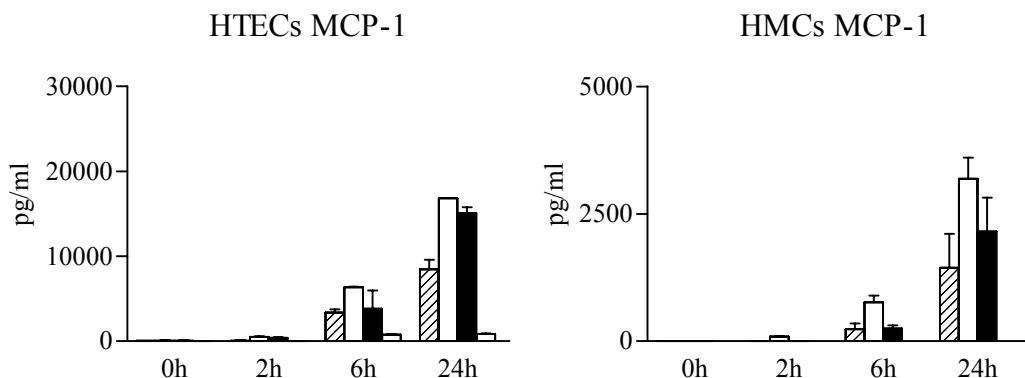


Figure 18 and 19. MCP-1 production in the supernatants from HTECs and HMCs. Hatched bars represent *E. coli* infected, open bars cells incubated with IL-1 β and filled combined stimulation (*E. coli* and IL-1 β).

RANTES production was seen at 6 hours in HTECs stimulated with *E. coli* or IL-1 β . At 24 hours, RANTES production due to *E. coli* stimulation was lower than that observed due to both IL-1 β and combined stimulation ($P = 0.02$ and $P = 0.002$ respectively) (Fig 20). In HMCs the RANTES production due to *E. coli* stimulation was higher than that seen due to IL-1 β stimulation ($P = 0.02$). Combined stimulation yielded additive increases in both HTECs and HMCs ($P < 0.001$) (Fig 21).

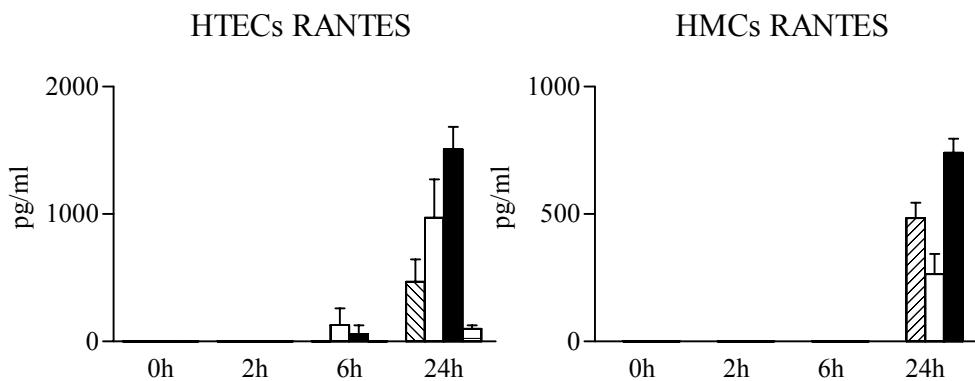


Figure 20 and 21. RANTES production in the supernatants from HTECs and HMCs. Hatched bars represent *E. coli* infected, open bars cells incubated with IL-1 β and filled combined stimulation (*E. coli* and IL-1 β).

Discussion

In experimental acute *E. coli* pyelonephritis, we have studied the expression of pro-inflammatory and down-regulating cytokines together with the chemokines: MIP-2/IL-8, MCP-1 and RANTES. We also investigated the influence of IL-6 and treatment with an Ang II type 1 receptor antagonist in this model.

In short we found a rapid increase in mRNA expression for pro-inflammatory and down-regulating cytokines both locally in the kidneys and systemically as observed in the spleens of mice with acute experimental *E. coli* pyelonephritis. Non-infected mice with experimentally induced urethral obstruction demonstrated similar changes in cytokine expression, although at a lower level. We also demonstrated an increased in the expression and secretion of MIP-2 (the IL-8 homologue in mouse), MCP-1 and RANTES. The same chemokines were also increased *in vitro* after *E. coli* stimulation of human tubular epithelial and mesangial cells. When mice were treated with an Ang II type 1 receptor antagonist, losartan, a significant reduction in TGF- β mRNA and protein levels was observed. Likewise IL-6 deficient mice had significantly lower renal TGF- β levels not only after *E. coli* infection, but also after inoculation with saline and acute urethral obstruction. When splenocytes from IL-6 deficient mice were exposed to rIL-6, TGF- β levels increased.

Pro-inflammatory mediator response in pyelonephritis:

The pro-inflammatory cytokines are thought to play important roles in the pathogenesis of pyelonephritis. In particular, IL-6 which at high concentrations acts to promote the progression of the disease, but at low levels appears to be involved in regulating repair processes (Kayama et al., 1997).

We have shown that the pro-inflammatory cytokines IL-1 β , IL-6, IFN- γ and TNF- α , and the chemokines MIP-2, MCP-1 and RANTES are up-regulated in infected animals. Experimentally a rapid pro-inflammatory cytokine response was found as early as 2 and 12 hours after bacterial inoculation particularly for IL-6. Our results extend previous observations that showed a rapid pro-inflammatory response locally in the kidneys in animals with *E. coli* pyelonephritis (Rugo et al., 1992) (Tullus et al., 1996) (Kabore, Simard, and Bergeron, 1999) (Hang et al., 1999).

Up-regulation of these mediators has also been reported in clinical pyelonephritis and IL-6 and IL-8 has been found in high concentrations in the urine of children after the acute phase of infection (Tullus et al., 1994a) (Jantausch, O'Donnell, and Wiedermann, 2000).

Urine concentration of IL-6 correlated with abnormal findings on DMSA scans performed in children after the acute phase of infection during infancy (Roilides et al., 1999) and after one year (Tullus et al., 1994b) and also three years after VUR had been surgically corrected (Wang et al., 2001). These data suggest that IL-6 may be involved in the pathogenesis of renal damage complicating pyelonephritis.

Obstruction and renal inflammation:

It has long been known that disturbances in urine flow are important in the development of UTI and especially acute pyelonephritis. In some children organic or functional bladder dysfunction may cause high urinary tract pressures in the storage phase or during voiding. This may produce frequent, intermittent obstructions of the ureter at the level of the bladder and thus facilitate the transfer of bacteria from the bladder to the kidneys (Vega and Pascual, 2001). Incomplete voiding of the urine due to obstruction makes it more difficult to eradicate bacterial infection and increases VUR. Experimental UUO or transient urethral obstruction *per se* has been shown to exacerbate urine stasis and induce foci of medullary necrosis. These changes then provide a nidus for bacterial invasion (Johnson et al., 1993) (Bitz et al., 2001).

We have found that obstruction not only is important in the initiation of infection, but also for the development of the cytokine response. Even a short duration of urethral obstruction (6 hours) produced cytokine and chemokine responses that were qualitatively similar to those of acute infection, albeit at lower levels. Obstructive injury has previously been shown to promote an inflammatory response in the interstitium with infiltration of mononuclear cells and proliferation of interstitial fibroblast (Wright et al., 1996).

Of great interest was also the prolonged expression of several cytokines especially TGF- β and MCP-1 found in our study. Likewise, an up-regulation of MCP-1 and TGF- β mRNA expression was shown in rat kidneys long after the removal of UUO (Wright et al., 1996) (Sommer et al., 2000). Obstruction *per se* might thus have an

influence on kidney physiology and function long after it has been alleviated. Even short and/or intermittent periods of obstruction might therefore be detrimental.

The similarities between the responses initiated by infection and by obstruction increases our understanding of the hazards associated with kidney obstruction as a complication associated with UTI.

Mechanism and development of renal scarring:

Several important steps characterize the process of inflammation and scarring. After bacterial attachment, IL-8 production is initiated and neutrophils are brought to the site of inflammation. Other chemokines, chemoattractants and adhesion molecules are also important at this stage. Acute pyelonephritis is characterized by renal parenchymal inflammation with extensive tubular destruction (Heptinstall, 1998). The acute inflammatory response is partially responsible for renal tissue destruction because of the respiratory burst of phagocytosis (Mundi et al., 1991). Cytokines and growth factors are released by damaged tubules and invading granulocytes. Parenchymal collapse results from tubular atrophy, loss of vascular, glomerular and interstitial volume. Since the tubulointerstitium constitutes of a major portion of the renal parenchyma, the parenchymal collapse is a product of the reduction in the tubular and interstitial area (Hewitson et al., 1998).

Later growth factors especially TGF- β become important for scar development. The metalloproteinases (MMPs) and tissue inhibitors of metalloproteinase (TIMPs) are also thought to be involved. TGF- β stimulates an ECM deposition by resident interstitial cells. Renal scarring is due to a disproportionate increase in ECM, resulting from an imbalance between matrix synthesis and degradation (Eddy, 2000).

Role of chemokines in tissue damage:

Interstitial leukocyte infiltration is a prominent early feature of tubulointerstitial inflammation and fibrosis. At the site of infection, chemokines and their specific receptors mediate accumulation of granulocytes which rapidly become active phagocytes (Segerer, Nelson, and Schlondorff, 2000). Neutrophil recruitment to the site of infection has been shown to depend on IL-8 and their cognate receptors. Urinary IL-8 supports neutrophil migration *in vitro* (Haraoka et al., 1999) (Hang et

al., 1999) and urinary IL-8 is increased in patients with pyelonephritis and correlated with leucocyturia (Tullus et al., 1994a)

Uroepithelial cells express CXCR1 and CXCR2, which are up-regulated by infection. Antibodies to the CXCR1 blocked the *in vitro* transepithelial migration of neutrophils (Godaly et al., 2000). In IL-8 receptor knockout mice with acute pyelonephritis, neutrophils were recruited to the kidneys but failed to cross the mucosal barrier to the lumen and accumulated in the subepithelial tissue. These mice were unable to clear the infection and showed renal scarring (Hang et al., 2000). Both MCP-1 and RANTES recruit inflammatory cells and have been shown to be critically involved in the development of interstitial fibrosis (Segerer, Nelson, and Schlondorff, 2000). In our experimental *E. coli* pyelonephritis, infected mice showed both mRNA expression and protein production of MIP-2, MCP-1 and RANTES that was sustained over the 6 day study period.

Role of neutrophils in the initiation of tissue damage:

Pro-inflammatory cytokines stimulate the expression of adhesion molecules, causing adhesion and migration of leukocytes to the site of infection where phagocytosis begins and where capillary obstruction may occur. This adhesion, which is followed by penetration of the capillary walls and entry into the interstitium and renal tubular space, results in granulocytic aggregation (Kaack et al., 1986).

Several previous studies showed that the inflammatory response itself could be responsible not only for bacterial destruction but also for the tissue damage to the host apart from any direct damage due to the bacteria itself. Tissue damage occurs when bacteria are phagocytized by neutrophils with concomitant release of lysosomal enzymes and free radicals such as superoxide, hydroxyl radicals and hydrogen peroxide into the surrounding renal tubular cells (Roberts et al., 1982).

It has also been shown that the extent of renal damage from the respiratory burst of phagocytosis directly correlates with neutrophil number and that this could be modulated by treatment with corticosteroids (Ormrod D, 1986). When the inflammatory response was experimentally prevented by decreasing chemotactic agents and thus decreasing phagocytosis, the acute renal damage was prevented (Roberts et al., 1983).

In animals with acute pyelonephritis treatment with colchicine, indomethacin, superoxide dismutase, allopurinol or a single dose of cyclophosphamide depressed the

leukocyte locomotion, chemotaxis, induced neutropenia and diminished lysosomal degranulation. These effects were shown to decrease both the inflammatory response and renal scarring, suggesting that the respiratory burst of bacterial phagocytosis played some role in the process of renal scarring due to bacterial infection. (Roberts et al., 1982) (Bille and Glauser, 1982; Glauser et al., 1983) (Roberts et al., 1986) (Matsumoto et al., 1990a) (Matsumoto et al., 1991).

While most cells in the body have superoxide dismutase, which acts to ameliorate the effect of the toxic oxygen free radicals, there is no superoxide dismutase in urine or in phagocytes (Fridovich, 1978). Thus the respiratory burst acts unopposed, damaging both neutrophils and renal tissues. It has been shown that the reactive oxygen radicals stimulate fibroblast growth, thus promoting fibrosis and scar formation. It was also shown that capillary obstruction and renal ischemia develop due to granulocytic aggregation in renal inflammation (Kaack et al., 1986). This process probably facilitates tissue invasion by bacteria resulting in additional damage and scar formation. Superoxide may also be produced during reperfusion of renal ischemic tissue.

Early antibiotic treatment is still the most potent protective measure against renal tissue damage since it stops bacterial multiplication and thus prevents the early influx of leukocytes to the site of infection (Glauser, Lyons, and Braude, 1979) (Glauser and Bonard, 1982).

Role of TGF- β in the pathogenesis of renal scarring:

As a consequence of the cellular events caused by infection, tubulointerstitial cells express a series of molecules resulting in matrix accumulation. TGF- β is a prime fibrosis promoting molecules that plays a major role in the fibrogenesis stimulated by both resident kidney cells and infiltrating leukocytes (Eddy, 2000). The fibrogenic property of TGF- β is derived from multiple actions. Active TGF- β triggers several events that promote fibrosis including transformation of fibroblast into myofibroblast, chemotaxis of fibroblast and monocytes, formation of interstitial matrix proteins and delayed matrix degradation. TGF- β autoinduces its own production and amplifies its biological action (Border and Noble, 1997) (Strutz et al., 2001).

In this study, we demonstrated an early and sustained up-regulation of TGF- β mRNA expression in mice with acute pyelonephritis. Of particular interest, TGF- β expression

was also up-regulated in non-infected, obstructed mice but at a lower level than the infected animals, indicating an influence of the obstruction *per se*. Our data are supported by results from an experimental model of unilateral urethral obstruction, where it has been shown that the expression of TGF- β mRNA is elevated in obstructed kidneys (Kaneto, Morrissey, and Klahr, 1993) (Diamond et al., 1994). Increased mRNA expression and protein levels of TGF- β have also been documented in obstructed human kidneys. These increases correlate positively with interstitial inflammation, tubular atrophy and ECM deposition, indicating an important role for TGF- β in the pathogenesis of fibrosis in obstructed kidneys (Goumenos et al., 2001). Antibodies to TGF- β and antisense oligodeoxynucleotides significantly reduce renal interstitial fibrosis after UUO. This observation adds support for a direct influence of TGF- β on kidney damage (Miyajima et al., 2000b) (Isaka et al., 2000).

Regulatory effect of angiotensin II on TGF- β expression:

The renin-angiotensin system has been suggested to have a major role in the pathogenesis of tubulointerstitial fibrosis in many different kidney diseases including obstructive nephropathy. This is probably mediated by increased levels of Ang II in renal tissue following obstruction (Ishidoya et al., 1995). Ang II is a potent vasoconstrictor that has growth and matrix promoting action that may contribute to local ischemia (Johnson, 1997). The interaction between the renin-angiotensin system and TGF- β might explain the particular susceptibility of the kidney to fibrosis (Border and Noble, 1998).

TGF- β mRNA was increased in the obstructed kidney in a rat model of UUO. This increase was partially reduced by administration ACE inhibitor and a significant reduction of the relative volume of the cortical interstitium was noted. A similar effect was seen after administration of an Ang II type 1 receptor antagonist (Ishidoya et al., 1995) (Pimentel et al., 1995), but monocyte/macrophage infiltration was not affected (Klahr and Morrissey, 1997). In our experimental model of *E. coli* pyelonephritis, we demonstrated a marked reduction of TGF- β and decreased cortical scarring in mice treated with losartan, an Ang II type 1 receptor antagonist. These results suggest that losartan therapy may be beneficial in reducing renal scarring during the late phase.

The role of ACE gene polymorphism in renal scarring:

Support for a role of the renin angiotensin axis in the development of renal scarring in human is also obtained from studies on the importance of ACE gene polymorphism. ACE is one of the key enzymes in the renin angiotensin system. The ACE gene displays an insertion/deletion polymorphism. Three genotypes are determined by the presence (insertion I) or absence (deletion D), II, ID and DD. High plasma and tissue levels of ACE are found in individuals with homozygous DD genotype (Rigat et al., 1990).

The ACE DD genotype has been found to have a significant impact on renal damage (Ozen et al., 1999). An excess of the DD genotype was found in children with congenital urological abnormalities and renal scarring. On the other hand, in patients with uropathy without parenchymal damage, no difference was observed compared to the normal population (Brock et al., 1997). ACE DD genotype was also identified as a risk factor for the development of renal damage associated with VUR (Hohenfellner et al., 1999). In line with this observation, a significant correlation was seen between serum ACE levels and DD genotype in patients with VUR (Ozen et al., 1997). Thus genotyping may be beneficial to identify children at risk for developing renal scarring.

The influence of IL-6 on bacterial clearance:

IL-6 is a multifunctional cytokine with pro-inflammatory and immunoregulatory functions. It has been shown that higher concentrations of urinary and serum IL-6 are found in patients with acute pyelonephritis than in those with asymptomatic bacteriuria (Hedges et al., 1992).

IL-6 deficient mice are unable to induce neutrophilia in response to both Gram positive (*Listeria monocytogenes*) (Kopf et al., 1994) (Dalrymple et al., 1995) and Gram negative (*E. coli*) infections (Dalrymple et al., 1996). IL-6 deficient mice also clear the infection from the liver and the spleen less efficiently and are more likely to succumb to the infection than are controls. Defective leukocyte recruitment to the site of inflammation in IL-6 deficient mice is associated with defective chemokine production. (Romano et al., 1997). Consistent with these observations, we also showed that in experimental acute *E. coli* pyelonephritis, IL-6 deficient mice retained viable bacteria in their kidneys longer than their wild type counterparts and IL-6 deficient mice were also more likely to die from *E. coli* infection. Administrations of recombinant IL-6 has been shown to restore neutrophilia in IL-6 deficient mice and

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reverse the accumulation of *E. coli* in the liver. Neutropenic mice also find *E. coli* infection to be fatal (Dalrymple et al., 1996) indicating the importance of neutrophils for bacterial clearance.

Conclusions

Cytokines were up-regulated both locally in the kidneys and systemically in the spleen in experimental acute *E. coli* pyelonephritis and also in non-infected but obstructed mice, indicating that both infection and obstruction *per se* influences the cytokine expression.

An angiotensin II type 1 receptor antagonist, losartan, reduces TGF- β and the pro-inflammatory cytokines IL-6 and IFN- γ . A slight reduction in cortical scarring was also seen.

Low TGF- β expression together with an extensive growth of *E. coli* in the kidneys and increased mortality was observed in the IL-6 deficient mice. This indicates an importance of IL-6 for prevention against bacterial infection and host survival.

MIP-2, MCP-1 and RANTES levels were found increased in kidneys during experimental pyelonephritis. Similarly IL-8, MCP-1 and RANTES were induced from the human renal tubular epithelial cell line and the primary human mesangial cells after *E. coli* or IL-1 β stimulation. An additive effect was observed on IL-8 and RANTES.

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