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Grönberg Hernandez, Jenny

2013

Link to publication

Citation for published version (APA):
Grönberg Hernandez, J. (2013). *Mechanisms of microbial-host interaction during asymptomatic bacteriuria*. [Doctoral Thesis (compilation), Division of Microbiology, Immunology and Glycobiology - MIG]. Division of Microbiology, Immunology and Glycobiology - MIG.

Total number of authors:
1

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Mechanisms of microbial-host interaction during asymptomatic bacteriuria

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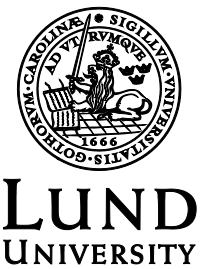

DOCTORAL DISSERTATION
With the permission of the Medical Faculty at Lund University, the defense of the thesis will take place on the 21st of September 2013 at 9:00 in Rune Grubb lecture hall, BMC, Lund.

Faculty opponent

Professor Harry Mobley, University of Michigan, USA

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Document name
DOCTORAL DISSERTATION

Date of issue September 21st, 2013

Author(s) Jenny Grönberg Hernández

Sponsoring organization Lund University

Title and subtitle Mechanisms of microbial-host interaction during asymptomatic bacteriuria

Abstract

Urinary tract infections (UTIs) present an interesting and relevant model for studying microbial adaptation. After establishing significant numbers, the bacteria either cause severe disease, or an asymptomatic carrier state resembling the normal flora at other mucosal sites. Patients with asymptomatic bacteriuria (ABU) are protected from re-infection if the strain that they carry outcompetes more pathogenic strains. Deliberate inoculation with the prototypic ABU strain *Escherichia coli* 83972 has therefore been developed and clinically proven to protect against recurrent UTI.
To define the host influence on bacterial adaptation during long term *E. coli* 83972 ABU, we collected sequential isolates from patients that had been inoculated with *E. coli* 83972 and established stable bacteriuria. The isolates acquired several host-specific mutations, demonstrating that *E. coli* 83972 adapts to the individual host. Each host provided a unique niche, which was demonstrated by significant variations of mucosal host response parameters between patients. Variation in the host response to ABU has lead to uncertainty about the use of host response parameters as a basis for diagnostic and therapeutic decisions. In 23 patients, the host response to *E. coli* 83972 was accompanied by a low but host-specific increase in neutrophil chemotaxis but IL-6 levels did not increase. To define the effects of genetic variation on the urine proteomic host response, patients were genotyped for polymorphisms that have been linked to susceptibility to ABU and urine samples from the patients were screened for 31 immune markers. The genetic polymorphisms in the interferon regulatory factor 3 (*IRF3)* and Toll-like receptor 4 (*TLR4)* promoter had a significant impact on the magnitude of the host response during *E. coli* 83972 ABU, which consisted of mainly innate immune mediators. The transcriptional host response to ABU has not been examined in humans. To examine if ABU strains affect uroepithelial and leukocyte human host gene expression, we analyzed peripheral blood leukocytes from patients colonized by *E. coli* 83972 and uroepithelial cells stimulated with the same strain. *E. coli* 83972 inhibited RNA Polymerase II phosphorylation and suppressed pathogen-specific pathways in both systemic leukocytes and uroepithelial. We show that ABU is an active rather than passive process, and present a theory that basal transcriptional suppression may be a general mechanism used by commensal strains to modulate host gene expression. We further examined the kinetics of the local and systemic host response during ABU and the effect of type 1 and P fimbrial adhesion on the transcriptional signature. All patients inoculated with *E. coli* 83972*pap* activated the Interferon signaling pathway. Two patients inoculated with *E. coli* 83972*fim* downregulated Natural Killer cell signaling. Our results provide direct molecular evidence of host specific evolution of bacterial genomes as well as transcriptomic alterations in the host during ABU.

Key words Asymptomatic bacteriuria, Urinary tract infection, Escherichia coli 83972, IRF3, TLR4, Evolution, host response, promoter polymorphisms, transcription, P fimbriae, Type 1 fimbriae

Classification system and/or index terms (if any)

Supplementary bibliographical information

Language English

ISSN and key title 1652-8220

ISBN 978-91-87449-75-8

Recipient's notes

Number of pages

Price

Security classification

Signature _____ Date 26/8-13

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Faculty of Medicine, Department of MIG
ISBN 978-91-87449-75-8
ISSN 1652-8220

Printed in Sweden, Lund University
Lund 2013

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II. Genetic control of the variable innate immune response to asymptomatic bacteriuria. **Grönberg Hernández J**, Sundén F, Connolly J, Svanborg C, Wullt B. **PLoS One. 6(11): 2011**

III. Bacterial control of host gene expression through RNA Polymerase II. Lutay N, Ambite I, **Grönberg Hernandez J**, Rydström G, Ragnarsdóttir B, Puthia M, Nadeem A, Zhang J, Storm P, Dobrindt U, Wullt B, and Svanborg C. **J Clin Invest. 2013 May 24**.

IV. Conversion from ABU to virulence: Effects of P and Type 1 fimbriae on human gene expression, signal transduction and symptoms. **Grönberg Hernandez J**, Ambite I, Ragnarsdottir B, Kovecz B, Zdziarsky J, Lutay N, Dobrindt U, Wullt B and Svanborg C. ***Manuscript***.

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ABBREVIATIONS

ABU	Asymptomatic bacteriuria
AMP	Antimicrobial peptide
APN	Acute pyelonephritis
BRD4	Bromodomain protein 4
cAMP	Cyclic adenosine monophosphate
CCL	Chemokine (C-C motif) ligand
CD48	Cluster of Differentiation 48
CDKN2A	Cyklin-dependent kinase inhibitor 2A
CLR	C-type lectin receptor
CNF1	Cytotoxic necrotizing factor
CREB	cAMP responsive element binding protein
CRP	C reactive protein
CTD	C terminal domain
CXCL	Chemokine (C-X-C motif) ligand
CXCR1	Chemokine (C-X-C motif) receptor 1
DAM	Deoxyadenoside methylase
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
ELR	Glutamic acid-leucine-arginine sequence
GSL	Glycosphingolipid
GTPases	Guanosine triphosphate
HDAC	Histone de-acetylase
IBCs	Intracellular bacterial communities

ICAM	Intercellular adhesion molecule 1
IFN	Interferon
Ig	Immunoglobulin
IKKε	Inhibitor κB kinase
IL	Interleukin
IL-1RA	IL-1 receptor antagonist
IRAK-1	IL-1 receptor-associated kinase 1
IRF	Interferon regulatory factor
ISGF3	Interferon-stimulated gene factor 3
LB	Luria broth
LBP	LPS binding protein
LILRB1	Leukocyte immunoglobulin-like receptor
LPS	Lipopolysaccharide
LRP	Leucin-responsive regulatory protein
LRR	Leucine-rich repeat
MAP	Mitogen activated protein
MAPKK	MAP kinase kinase
MBL	Mannose-binding lectin
MD2	Myeloid differentiation protein 2
mRNA	Messenger RNA
MYD88	Myeloid differentiation protein 88
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cell
NK cell	Natural killer cell
NLR	NOD-like receptor
NOD	Nucleotide-binding oligomerization domain
P-TEFb	Transcription elongation factor
PAI	Pathogenicity islands
PAMP	Pathogen-associated molecular pattern

pap	*Pyelonephritis associated pili*
PBL	Peripheral blood leukocyte
PLC	Phospholipase C
PMN	Polymorphonuclear neutrophil
PRR	Pattern recognition receptor
prs	*Pap-related sequence*
RIG1	Retinoic acid-inducible gene 1
RLR	RIG1-like receptor
RNA	Ribonucleic acid
RNA Pol II	RNA Polymerase II
RT-PCR	Real-time polymerase chain reaction
Sat	Secreted auto-transporter toxin
Scid	Severe combined immunodeficiency
Ser	Serine
Sis	ShiA-like inflammation suppressor genes
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
STAT1	Signal Transducer and Activator of Transcription
TANK	TRAF family member-associated NFKB activator
TBK	TANK binding kinase
TBK1	TANK binding kinase 1
TcpC	Tir containing protein C
THP	Tamm-horsfall protein
TIR	Toll/interleukin-1 receptor
TIRAP	TIR domain containing adaptor protein
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAF	TNF receptor associated factor
TRAM	Toll-like receptor adaptor molecule 2

TRIF	TIR-domain containing adapter-inducing interferon-β
UPEC	Uropathogenic Escherichia coli
UTI	Urinary tract infection
VUR	Vesicoureteral reflux
wt	Wildtype

INTRODUCTION

1. Urinary tract infections

Urinary tract infections (UTIs) are the most common bacterial infections worldwide [1,2] and affect individuals of all ages and social groups. The normal urinary tract is usually sterile due to robust host defense mechanisms. Bacteria frequently enter the lower urinary tract and if they persist, cause UTI. UTI may be localized to the lower (cystitis) or upper (acute pyelonephritis (APN)) urinary tract, and be symptomatic (cystitis and APN) or asymptomatic (asymptomatic bacteriuria (ABU)).

Fifty percent of all women experience at least one symptomatic lower UTI in their lifetime [3], and one third of these will suffer a recurrent episode within one year [4], the majority of these representing a re-infection [4].

More than 80 years after their discovery, antibiotics are still the most effective treatment against acute UTI, but due to increasing microbial multiresistance to antibiotics alternative treatments are urgently needed [5,6]. Antibiotics are significantly overused due to difficulties in diagnosis [7]. This is especially the case in patient groups with a high frequency of asymptomatic bacteriuia (ABU) such as elderly or institutionalized patients with multiple diseases. A recent review of studies in a hospital setting found that 20-52% of patients with ABU are treated unnecessarily with antibiotics [7].

Asymptomatic or symptomatic UTI

UTI is in the majority of cases (>70%) caused by uropathogenic *Escherichia coli* (*E. coli*) (UPEC) and occurs when strains from the perianal, intestinal or preputial microflora gain access to the urinary tract through the urethra [8-12].

In the majority of cases the bacteriuria is transient and sterility prevails. However, if the strain persists, it will multiply to colonize the lower urinary tract. Once the strain has established itself, the interaction between the microbe and the host defenses determines the outcome of the infection.

Frequently, the bacteriuria results in ABU, defined as asymptomatic carriage of $>10^5$ bacteria/ml urine detected in two subsequent urine cultures [13]. ABU occurs in 1% of schoolgirls [14] and is estimated to 3.5% in the general population [3]. Prevalence is increased

to 3.6-19% in otherwise healthy elders [15,16], 0.7-27% in patients with Type 1 diabetes, 1.9-9.5% in pregnant women [17] and 23-89% in patients with spinal cord injuries [18].

ABU is often accompanied by a low mucosal host response resulting in increased urine leukocytes and cytokine levels [13,19]. While urine cytokine levels may increase, no increase in IL-6 or IL-8 in serum is detected [19-22].

ABU is known to persist for weeks up to years without causing symptomatic or renal deterioration [23,24]. ABU can protect against superinfection by more virulent strains that are capable of causing symptomatic infection [25-27] (this subject is further discussed in Chapter 2). Therefore ABU should not be treated by antimicrobials, unless complicating physiological factors are present [28]. Pregnant women are an exception [17] as untreated ABU is a risk factor for subsequent pyelonephritis and low birth-weight infants [29].

Acute, uncomplicated cystitis is localized to the lower urinary tract in the pediatric and adult population. It is most common in otherwise healthy, non-pregnant, premenopausal women with normal urinary tracts. Cystitis occurs in 30% of all women before the age of 24 [30], and sexual debut is the primary risk factor [31-33]. Symptoms are mostly a combination of urgency, lower abdominal pain and dysuria [34] accompanied by a mucosal immune response leading to increased urine leukocytes, cytokine levels and in some cases hematuria. Recurrent infections are common and risk factors are the same as for single episodes [4,32].

APN occurs when virulent bacteria ascend through the urethra to the renal pelvis. It is the most serious form of UTI, as it may affect kidney function and in the worst cases cause septic shock. Symptoms are fever, flank pain, urgency and general malaise. The host response engages both the mucosal and the systemic compartments resulting in local and systemic inflammation detected by fever, increased levels of C reactive protein C (CRP) in serum and leukocytosis [35,36].

Susceptibility to UTI

Susceptibility to UTI is inherited, as described by both family studies and gene analysis [37,38]. Specific polymorphisms predisposing for ABU [39,40], APN [37,41] and kidney scarring in children with vesicourethral reflux (VUR) [42,43] have been identified. Predisposition to ABU has been linked to promoter polymorphisms in *Toll-like receptor (TLR) 4* [40] and single nucleotide polymorphisms (SNPs) in *TLR2* and *chemokine (C-X-C motif) receptor 1 (CXCR1)* [39].

C3H/HeJ mice, which have a point mutation in *Tlr4*, develop an ABU-like state after *E. coli* bladder inoculation [44] and children who are prone to ABU have lower TLR4 expression [45]. ABU-prone patients have *TLR4* promoter polymorphisms, which decrease *TLR4* promoter activity [40].

The *TLR2* polymorphism impaired the innate immune response *in vitro* [39] and correlated with gram-positive bacteriuria [46], and the *CXCR1* variants were associated with changes in IL-8 levels in women with ABU [39]. Polymorphisms in *interferon regulatory*

factor 3 (IRF3) were initially discovered in patients with systemic lupus erythematosus (SLE) [47], but are also linked to susceptibility to ABU and APN [41]. ABU-susceptible individuals carried a heterozygous variant of the allele (-925, -776), which increases *IRF3* promoter activity [41]. In of the murine UTI model, *Irf3-/-* mice develop severe acute pyelonephritis with urosepsis and rapid kidney abscess formation, suggesting a protective effect of IRF3 or resulting effector functions in acute pyelonephritis [41].

Susceptibility to APN is also influenced by the patient's blood group [48]. Individuals with the P_1 blood group are more prone to developing APN and carry more P fimbriated strains in their fecal flora compared to P_2 individuals [11]. ABO non-secretors with APN are more susceptible to kidney scarring [49].

UTI is more common in females than in males, except after 70 years of age. In children <6 years the cumulative incidence rate of first-time UTI is 3 times greater in girls (6.6%) than boys (1.8%) [50]. In addition, early age at onset of UTI is a risk factor for recurrencies in girls.

Other factors which influence UTI susceptibility are vaginal microflora [51,52], anatomy [31] age, and diet [53]. Anatomical or functional abormalities such as incomplete bladder emptying due to infravesical outflow obstruction (e.g. as a result of prostate hypertrophia), neurogenic bladder disorders or bladder stone formation predispose to UTI by restricting the urine flow or serving as reservoirs for incoming strains. Immunosuppression, nosocomial factors and instrumentation also facilitate the establishment of UPEC in the urinary tract.

2. Asymptomatic bacteriuria

Historically there was a concern that ABU would cause chronic pyelonephritis and end stage renal disease with uremia. During the period between 1950 and 1980 the first studies were published that examined the microbiology and immunology of ABU, which in the late 1980s could be identified as beneficial in patients without risk factors.

The first screenings for ABU

In the 1950s, UTI was diagnosed by its clinical manifestations and verified by positive urine cultures. However, medical practitioners were encountering asymptomatic patients with positive urine cultures and *vice versa*, and needed diagnostic definitions to distinguish between "true bacteriuria" and contamination to ensure accurate treatment. This was determined in 1956, when Kass demonstrated that $\geq 10^5$ cfu/ml urine, later referred to as "significant bacteriuria", will predict bacteriuria in a subsequent sampling occasion in asymptomatic females [13]. Kass and Sandford concluded that symptoms but

also pyuria, which were used to diagnose APN, only correlate with bacteriuria in a fraction of patients [13,54]. At this time APN was the most common complication in pregnant women, and screening studies of pregnant women showed that ABU was preceeded APN in 40% of cases [55].

To establish the clinical effects of ABU and the prevalence in the general population, an ambitious four-year screening study was performed in Virginia, USA in the early 1960s [56]. Schoolchildren were screened for bacteriuria, and ABU was detected in 1.1% of all girls [56]. Similar results were obtained in a study of 5-year-old children from Dundee, Scotland [57]. These first studies established that ABU was more common than symptomatic UTI.

Change in perspective: the beneficial infection

It was not until epidemiological studies of ABU in the 1970s that ABU was clinically separated from other forms of UTI. Antimicrobial treatment is effective in removing ABU, due to recurrent infections there is no significant difference in cure rate between treated and un-treated patients one year after treatment [58]. In several papers Lindberg examined ABU in schoolgirls (7-15 years old). He investigated the level of pyuria and C-reactive protein (CRP) [14], the difference between symptomatic isolates and ABU isolates [59], the relevance of residual urine [60] and the fecal flora [10].

In a three-year follow-up of girls that had been treated or not treated for ABU, Lindberg found no significant difference in the prevalence of bacteriuria [23]. Almost a decade later, Hansson performed a controlled trial [25] of untreated versus treated ABU. In 15% of the treated patients, symptomatic recurrences occurred and the majority of cases (seven out of eight) resulted in APN [25]. In contrast, none of the patients that remained bacteriuric developed symptomatic UTI [25], indicating that antimicrobial treatment of ABU predisposes for APN. The girls with un-treated ABU did not develop decreased renal concentrating capacity [59] or negative effects on excretory urography and micturition cystourethrography [23].

Lindbergs thorough, populationbased study and Hanssons controlled trial gave strong enough evidence to suggest against antimicrobial treatment in girls with ABU and normal urinary tracts [23,25] and further studies have verified the protective effect in adults [61].

Bacterial interference

In view of the protective effects of ABU, several different approaches have been taken to establish and ABU like, protective states in UTI prone patients. The inoculation with *E. coli* isolates from the patient's own fecal flora was successful and the bacteria

persisted for up to four months [62]. Similar beneficial results have been obtained with the prototype ABU strain *E. coli* 83972 (see below).

The commensal flora protects its host against pathogens, and several subspecies of lactobacilli colonize the healthy vaginal tract and the urethra. Females with recurrent UTI have lower lactobacilli counts and topical application of lactobacilli can reduce the UTI incidences by reestablishing a healthy flora [52]. The same effect can be achieved by using estrogen treatment in post-menopausal women [63].

In vitro, lactobacilli aggregate with *E. coli* [64] and inhibit adhesion of UPECs to human uroepithelial cells [65]. The presence of one strain in the urinary tract may exclude the establishment of another [24,66,67], suggesting a beneficial effect for individuals prone to recurrent UTI of carrying an avirulent strain. Instillations of lactobacilli into the human urinary tract were unsuccessful in persisting [52], but inoculation with *E. coli* isolates from the patient's own fecal flora persisted for up to four months [62].

E. coli 83972: a prototypical ABU strain

E. coli isolates from patients with ABU differ from the strains that cause symptomatic infections. ABU strains adhere poorly to human uroepithelial cells [68-70], are more sensitive to the bactericidal effects of serum [59] and have different O group distribution and K antigens [59,71]. ABU strains seldom express the adhesive P fimbriae or hemolysins, which are expressed by the vast majority of APN strains [72-77].

Despite ABU strains not being able to express several virulence factors, 60% of ABU strains carry virulence genes commonly found in UPEC strains [78 75]. ABU strains accumulate mutations and deletions in virulence genes, suggesting an ongoing adaptation from UPEC to ABU [79]. ABU strains are genetically related to either UPEC strains, but have smaller genome sizes and non-functional virulence genes, or a second category, which resembles fecal or avirulent strains [79].

E. coli 83972 was isolated during a screening study for ABU in schoolgirls [14]. The strain had been carried by a schoolgirl for at least three years without causing any renal deterioration or subjective symptoms from the urinary tract. *E. coli* 83972 belongs to the B2 lineage and the clonal sequence type ST73 [79]. This is the same clonal group as the APN isolate CFT073. Indeed *E. coli* 83972 carries defective genes coding for type 1, P and F1C fimbriae amongst other virulence factors [69,79,80].

E. coli 83972 inoculation protects against symptomatic UTI

Human therapeutic inoculation with *E. coli* 83972 was first used in 1991 by Andersson *et al.* [24] with the purpose of establishing prophylactic *E. coli* 83972 ABU in patients with recurrent UTI. The protocol uses *E. coli* 83972 cultured overnight in Luria broth (LB). The bacterial cells are washed in saline, and diluted to 10^5 cfu/ml, and 30 ml

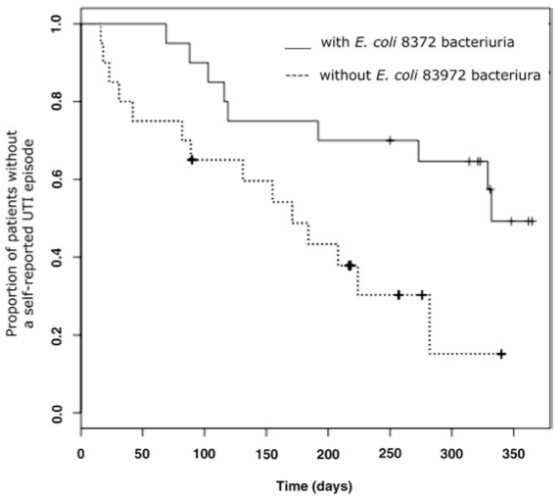

Figure 1. Risk of UTI in 20 patients (Kaplan-Meier estimate). There was significant delay to UTI in patients with *E. coli* 83972 bacteriuria, compared to when same patients were without *E. coli* 83972 bacteriuria. Patients were randomized to *E. coli* 83972 or saline inoculations, and were followed for 12 months or until self-report of UTI [26].

is inoculated into the urinary bladder via a cathether, which is then removed. Stable bacteriuria without increase in CRP, systemic leucocyte counts or erythrocyte sedimentation rate is established after the inoculation and the patients remain asymptomatic [24]. Prior to inoculation, preexisting bacteriuria is eradicated by antimicrobial treatment, which increases the colonization success rate [81,82].

The protection against recurrent UTI by bladder inoculation of *E. coli* 83972 has been clinically proven in intra- and inter-individual settings in Sweden [26,83] and USA [82,84-86]. In 67% of the patients *E. coli* 83972 ABU persisted for more than three months, and in three patients the strain remained for up to four years [83]. The success rate of the inoculations vary with the patient group characteristics, but at least 2/3 of the inoculations result in more than one month of bacteriuria [82-85].

E. coli 83972 inoculations protect patients against symptomatic UTI [26]. A double-blinded, randomized cross-over placebo-controlled study compared the time to symptomatic UTI after *E. coli* 83972 or saline inoculations [26]. The results demonstrated a significantly longer time to symptomatic UTI after *E. coli* 83972 inoculations (Figure 1). The protective effect was identical for patients with spinal injuries, residual urine, males and females [26].

The protective effect has also been confirmed in placebo-controlled studies of *E. coli* 83972 inoculations in a male population [85], using an *E. coli* 83972 derivative (HU2117) which lacks the entire *PapG* gene [27].

E. coli 83972 is well adapted to growth in human urine, and outcompetes UPEC strains during growth in human urine *in vitro* [87]. The biological barrier effect of *E. coli* 83972 may be useful to avoid colonization of virulent bacteria on catheters, as the majority of catheterized patients develop bacteriuria. Coating catheters with a layer of *E. coli* 83972 to reduce the overgrowth of gram-negative and fungal uropathogens have been successful *in vitro* [88,89] and *in vivo* [86]. Bacterial interference and adherence of *E. coli* 83972 to the catheter surface is improved by transformation of *E. coli* 83972 with *fim*+ plasmids, which code for type 1 fimbriae [90]. The efficacy improved further when the cathether surface was coated with the type 1 fimbrial receptor [91].

Inoculation with transformed variants

The long symptom free periods during *E. coli* 83972 ABU are an important improvement for patients with recurrent symptomatic UTI. However, *E. coli* 83972 is only successful in persisting long-term in patients with incomplete bladder emptying due to e.g. lower motor neuron lesions and neurogenic bladder disorders [81 82]. Patients with complete bladder emptying and otherwise normal lower urinary tract function excrete the strain and cannot be long-term colonized [81]. *E. coli* 83972 does not adhere to bladder cells due to lack of functional adhesion factors (P, F1C and type 1 fimbriae [69]). Adherence to the uroepithelial mucosa is a critical factor for successful colonization of the urinary tract [72], and is mediated by thin hair-like organelles, fimbriae, expressed on the bacterial surface that bind to receptors on the uro-epithelial cells [72] (discussed in detail in Chapter 3). *E. coli* may express no, one or several types of fimbria at the same time.

In an attempt to increase the colonization fitness of *E. coli* 83972, patients were inoculated with *E. coli* 83972 transformants carrying plasmids with the gene clusters *pyelonephritis-associated pili* (*pap*), *pap-related sequence* (*prs*) or an empty vector [22,92,93]. The studies focused on the effect of P fimbrial adhesion on the establishment of bacteriuria and the host response, and showed that P fimbriated (*pap+/prs+*) transformants of *E. coli* 83972 establish bacteriuria more rapidly and require a lower number of inoculations to reach significant numbers [92]. P fimbriated *E. coli* 83927 reached levels of 10^5 cfu/ml urine within 0.8 days, indicating a role for P fimbriae in the very early establishment of bacteriuria [92]. The expression of P fimbriae *in vivo* has been debated, as subculture of isolates may affect phase variation. However, *E. coli* 83972 expressing P fimbriae adhered to exfoliated epithelial cells in urine from colonized patients [93] and agglutinated human erythrocytes [92].

Despite the efficiency of establishment by the P fimbriated *E. coli* 83972 transformant, the patients remained asymptomatic. P fimbriated transformants triggered higher levels of urine Interleukin (IL) 6, Chemokine (C-X-C motif) ligand 8 (CXCL8, also known as IL-8) and polymorphonuclear neutrophil (PMN) infiltration, which correlated with phenotypic expression of P fimbriae *in vivo* [22]. Further studies of PapG

mediated adherence demonstrated that it was the functional adhesin that mediated the effect [93].

The effect of P fimbriae on establishment and host response induction is not a general hallmark of adhesion factors. Inoculation studies using plasmid-transformed type 1 fimbriated *E. coli* 83927 have demonstrated poor adhesive ability on exfoliated uroepithelial cells and no change in establishment rate of bacteriuria [94]. The type 1 fimbriated *E. coli* 83972 did not provoke inflammation in the human urinary tract, since IL-6, IL-8 and PMN infiltration remained at the same levels as after inoculation with the wild type (wt) strain [94].

3. Bacterial virulence in the urinary tract

Bacterial virulence may be defined by the infectivity of the pathogen and the severity of the disease it causes. Genes that influence *E. coli* virulence are often organized in pathogenicity islands (PAI) originating from plasmid, bacteriophage or mobile element insertions. UPEC strains express a specific repertoire of PAIs, typically lipopolysaccharide (LPS) (O antigen), capsule (K antigen) and flagella (H antigen) [95]. Several virulence genes may act in synergy and both host and bacterial factors regulate their expression [96].

Adhesion factors are expressed by many UPEC strains [72,97]. The most common are P fimbriae and type 1 fimbriae, F1C/S fimbriae are less prevalent as are the afimbrial adhesion factors Dr and Afa [97].

P fimbriae

E. coli expression of P fimbriae has been demonstrated to correlate strongly to disease severity in pediatric and adult populations in terms of inflammation and localization [11,50,72-74,76,77,98,99]. The P fimbrial pap gene cluster is found in 70-91% of APN isolates, 30% of cystitis isolates, 7-19% of fecal isolates and 20-24% of subcultured ABU isolates [11,73,74,76,100,101]. In children with recurrent UTI the prevalence of P fimbriated strains in the fecal flora is higher than in controls, and the symptomatic infections that they suffer are caused by strains expressing P fimbriae [11].

The pap gene cluster consists of eleven genes, of which six (*papA*, *papC*, *papK, papE*, *papF* and *papG*) encode structural proteins. P fimbriae are composed of a rigid stalk of PapA proteins, and a tip fibrillum (PapE, PapF, PapK and PapG) [102,103]. The PapG adhesin mediates attachment to the uroepithelia through binding to Galα(1–4)β receptor epitopes in the globoseries of glycosphingolipids (GSLs) [104,105]. The receptor-GSLs are abundant in the uroepithelia [73], but vary depending on the patient's P blood group of the patient [106]. The receptor-GSLs are P blood group antigens (P_1, P_2, P^k) and their expression varies with the blood group of the individual. P fimbriae agglutinate P_1 and P_2 erythrocytes but not p erythrocytes, which lack the receptor-GSLs [107]. Individuals with blood group P_1 run a 17 fold higher risk of developing APN with P fimbriated bacteria than individuals of other blood groups [108] but individuals of blood group p are most likely highly resistant to APN. Their numbers are too low to address this question.

P fimbriae are classified according to their iso-receptor specificity. Four variants have been discovered to date, representing different alleles of the *papG* gene [99,109-111].

Class I P fimbriae carry $PapG_{J96}$ adhesin, which binds to globotriaosylceramide (GbO3). The allele is rare and the association to UTI is unknown. Except for in its original finding in J96, $PapG_{J96}$ has only been found in a group of archetypal extraintestinal pathogenic strains [112].

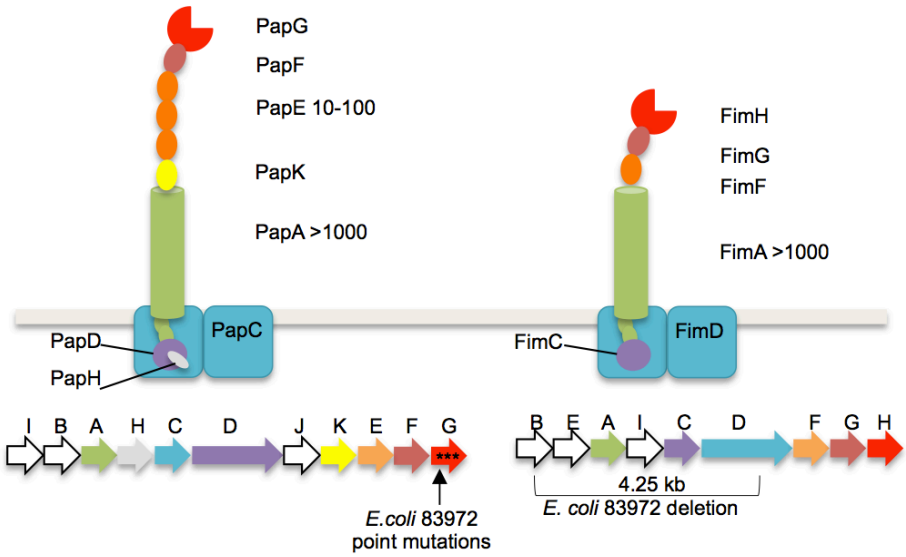

Figure 2. Structure of the P and type 1 fimbrial organelle and gene. *E. coli* 83972 has point mutations in the PapG adhesin and a 4.24 kb deletion in the fim gene cluster. Adapted from Remaut *et al.* [113].

Class II P fimbriae carry the $PapG_{IA2}$ adhesin, which binds to most members of the globoseries of GSLs or GbO4. Class II P fimbriae have a strong association to APN and bacteremia [114].

Class III P fimbriae carry the $PrsG_{J96}$ adhesin and bind to Forssman glycolipid that is present on sheep erythrocytes or GbO5. It is associated with cystitis in humans [115] and genitourinary tract infections in canines [116].

A fourth PapG allele was recently discovered during in a screening study of a large *E. coli* collection [111]. The receptor is still unknown. The gene it is most similar to the Class I allele.

P fimbriae adhere to the bladder epithelium, vessel walls, muscular layer and in the kidney to Bowman's capsule, glomerulus, the collecting duct, proximal and distal tubulus and the vessel walls [99,117].

In vitro P fimbrial phase variation is affected by temperature, osmolarity, pH and growth conditions [118]. Transcription of P fimbriae occurs via activation of the pBA promoter. It is regulated by the methylation of target sequences proximal to the pBA promoter by deoxyadenoside methylase (Dam) [119], and the binding of leucin-responsive regulatory protein (Lrp). Lrp may activate or repress transcription from the pBA promoter, depending on its binding site [120].

Human inoculation studies with functional P fimbriated transformants of the ABU strain *E. coli* 83972 have provided valuable insights into the isolated role of P fimbriae in the urinary tract. *E. coli* 83972 carrying genes coding for class II or class III fimbriae

establish bacteriuria faster than the parent strain [92] and induce higher levels of urine IL-6, IL-8 and PMN recruitment in the patients [98].

Type 1 fimbriae

Type 1 fimbriae are encoded by the *fim* gene cluster, which is present in the majority of fecal and uropathogenic *E. coli* isolates. The *fim* gene cluster consists of nine genes of which five (*fimA*, *fimI*, *fimF*, *fimG* and *fimH*) encode structural proteins [121]. Type 1 fimbriae are predominantly comprised of one major structural subunit (FimA) and a tip fibrillum (FimF, FimG, FimH). The FimH adhesin binds to mannosylated glycoproteins such as the Tamm-horsfall protein (THP) [122], integrins [123], cluster of differentiation 48 (CD48) on mucosal mast cells [124], IgA [125] and uroplakins IIIa and Ia [126]. In the urinary tract, type 1 fimbriae adhere to the vessel walls, muscular layer, collecting duct, proximal and distal tubulus [117].

Type 1 fimbriae promote virulence in murine models of UTI [127,128], but in the human urinary tract type 1 fimbriated *E. coli* 83972 did not trigger an inflammatory response or adhere to exfoliated epithelial cells [94]. In children with acute pyelonephritis due to the O1:K1:H7 clone, type 1 fimbrial expression correlated with disease severity [129]. However, type 1 fimbriae promote host cell invasion *in vitro* and form intracellular bacterial communities (IBCs) in the uroepithelia, which have been suggested to cause recurrent UTI [130-132].

FimH binding to Uroplakin IIIa induces elevated intracellular levels of Ca^{2+} followed by uroepithelial apoptosis and exfoliation [133], but also invasion in experimental models. IBCs seem to localize to fusiform vesicles in the uroepithelia, as chemically increasing intracellular cyclic adenosine monophosphate (cAMP) levels induced exocytosis of these and lowered the number of IBCs [134]. However, ABU strains often carry dysfunctional *fim* gene clusters and their involvement in IBC formation is therefore not certain [79].

The *fim* gene cluster is subject to phase shifting. The promotor lies within a short invertible element of deoxyribonucleic acid (DNA), and fimbrial structural genes are expressed when the element is in one orientation but not the other [135]. The inversion is catalyzed by the tyrosine recombinases FimE (which turns the gene off) and FimB (which turns the gene on) [136,137]. Type 1 fimbrial phase shifting is influenced by environmental factors such as growth medium and pH [138].

Biofilm and intracellular bacterial communities

Bacterial biofilm formation occurs in all types of aqueous or moist environments. In the urinary tract the formation of biofilm may protect bacteria against environmental stress, phagocytosis and antibiotics. In the absence of adhesive factors, biofilm may

increase the strains ability to persist. Biofilms consist of proteins, polysaccharides, DNA and several other factors and are can build up around implants, kidney stones and possibly intracellularly [139]. UPEC expressing type 1 fimbriae have been suggested to invade bladder cells to form biofilm-like IBCs [140]. Intracellular amplification occurs in "pods", superficial bladder epithelial cells, and these were detected in urine samples from women seeking treatment for UTI [141]. *In vitro*, 16% of cystitis isolates were capable of forming biofilm [100]. Furthermore, ABU isolates formed biofilm more readily than APN isolates *in vitro* [142].

Soluble virulence factors

Many UPEC secrete toxins that cause tissue damage, facilitate invasion and extract nutrients. Alpha-hemolysin is a pore-forming toxin, which causes cell lysis and intracellular signaling [143]. It increases clinical severity in patients and is present in 31-48% of UPEC strains [144], often in conjunction with cytotoxic necrotizing factor (CNF1). CNF1 activates Rho guanosine triphosphate enzymes (GTPases) in the host cell, promotes bladder cell exfoliation, intracellular signaling and counteracts phagocytic activity and chemotaxis of PMNs. CNF1 is present in 30% of UPEC strains [144]. CNF1 modifies PMN function by decreasing membrane fluidity, which has effects on complement receptor clustering and phagocytic function [145].

Tir containing protein C (TcpC) is present in 40% of clinical pyelonephritis isolates. It inserts into the host cell and inhibits the host response via myeloid differentiation protein 88 (MyD88)-dependent signaling pathways [146]. Secreted autotransporter toxin (Sat) is a serine protease toxin produced by UPEC that has cytopathic effects on kidney and bladder cells [147].

UPEC may also stabilize the nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κB) inhibitor κB (IκB), which increased bladder epithelial cell apoptosis [148]. In addition, shiA-like inflammation suppressor genes (Sis) SisA and SisB suppress host inflammatory responses, as deletion of these genes causes significantly higher levels of acute inflammation in a murine model of UTI [149].

To overcome iron limitation in the urinary tract *E. coli* also expresses a variety of iron scavenger systems known as siderophores. Aerobactin is frequent in UPEC isolates (present in 45% of symptomatic isolates [150]), but multiple systems may be expressed during colonization. The salmochelin siderophore receptor IroN promotes invasion of uroepithelial cells in vitro, which may indicate a dual function for the iron scavenger systems [151].

LPS is the major component of the outer membrane of Gram-negative bacteria and consists of a polysaccharide chain covalently bound to a lipid component (Lipid A). The full-length wild-type O-chain is termed "smooth", and the truncated version (due to mutations in genes involved in O-chain synthesis and attachment) is termed "rough" [152]. Injection of LPS from the NU14 *E. coli* strain, which expresses the "smooth" phenotype

mediated a pain response in mice which was not seen in mice injected with LPS from *E. coli* 83972, which expresses the "rough" phenotype [153].

ABU strains seldom express toxins. A comparison of clinical ABU, cystitis and APN isolates found that 14% of ABU strains had hemolytic activity, compared to 47% of the cystitis isolates [70], and *TcpC* was only found in 16% of ABU strains [146].

4. Activation of the host response in the urinary tract

Individuals with ABU carry the same number of bacteria as symptomatic patients, but exhibit a lower host response and do not develop symptoms [21]. The host response is triggered by the presence of virulence factors in the infecting strain [93], which activate specific parts of the host defenses.

Innate immunity acts fast and consists of a variety of proteins and receptors that recognize evolutionary conserved motifs in pathogens (pathogen-associated molecular patterns or PAMPs). Pattern recognition receptors (PRRs) that may be membrane-bound or cytoplasmic, and are divided into four groups: C-type lectin receptors (CLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene 1 (RIG1)-like receptors (RLRs) and TLRs. Activation triggers a myriad of actions, including release of chemotactic mediators and transcription of genes.

Adaptive immunity is mediated by several subsets of T cells, B cells and recently a subset of Natural killer (NK) cells have been reported to be implicated in the adaptive immune response [154]. Adaptive immunity is activated by innate immunity and foreign antigens and is highly specific, recognizing single epitopes on the immunogen. Activation results in antibody production, which may be long-lasting if memory cells are engaged.

Innate immunity in the urinary tract

The host cannot solely use ''pattern recognition'' through LPS and other conserved bacterial patterns, since these are present on both pathogens and commensals. Instead, pathogen-specific mechanisms are used by virulent uropathogenic *E. coli to* break the inertia of the mucosal barrier [41,155,156]. In the urinary tract activation of TLR4 is regulated via binding of Type 1 and P fimbriae to uroepithelial receptors [156,157]. To recognize the bacterial cell wall component LPS, TLR4 must associate with membrane-bound and soluble co-receptors which are not present in the uroepithelia [158-160].

TRIF-dependent signaling is activated by P fimbriated E. coli

In the urinary tract, TIR-domain containing adapter-inducing interferon-β (TRIF (also known as TICAM1))-dependent signaling through TLR4 is activated by P fimbrial binding to GSLs moieties on the surface of uroepithelial cells [93,156,157,161]. The GSLs are

anchored to ceramide, a simple sphingolipid that is found in lipid rafts, caveolae or anchoring signaling proteins. Ceramide can act in signal transduction when it is released from the membrane by cleavage by acid sphingomyelinase [162]. Cell stimulation with P fimbriated *E. coli* increases intracellular levels of free ceramide, activating a TLR4 dependent response [156], and results in nuclear translocation of IRF3 and NF-κB [41]. Briefly, TLR4 activates Toll-like receptor adaptor molecule 2 (TRAM, also known as TICAM-2), a membrane anchored protein that functions as a bridge between TLR4 and TRIF [163]. TRIF associates with tumor necrosis factor (TNF) receptor associated factor (TRAF) 3, interleukin-1 receptor-associated kinase (IRAK)-1, TRAF family member-associated NFKB activator (TANK) binding kinase (TBK) 1 and inhibitor κB kinase (IKK) ε, which phosphorylate IRF3. IRF3 then forms a complex with cAMP responsive element binding protein (CREB), and translocates to the nucleus to initiate transcription.

The pathogen-specific activation of TLR4 has been demonstrated in uroepithelial cells and murine models of UTI, where ceramide release induced phosphorylation of CREB, Fyn, phospholipase C (PLC) γ, mitogen-activated protein (MAP) kinases and AP1 [41].

MyD88-dependent signaling is activated by type 1 fimbriated E. coli

Type 1 fimbrial binding to mannosylated moieties on uroepithelial cells induces the MyD88 dependent pathway [41]. MyD88 is recruited to all TLRs except for TLR3. TLR activation results in recruitment of MyD88 by TIRAP, and subsequent recruitment of IRAK, which is phosphorylated and associates with TRAF6. TRAF6 interacts with two ubiquitin-conjugating enzymes and associates with TAK1 and TAK1-binding proteins. TAK1 phosphorylates the IKK complexes, which leads to degradation of IκB and release of NF-κB. TAK1 may also activate MAPKKs such as JNK (MAPK8), which induce nuclear translocation of AP1 [164].

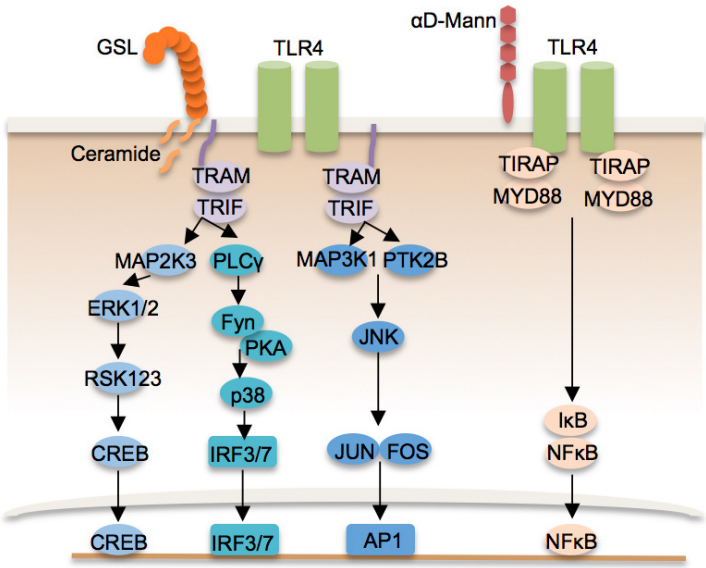

Figure 3. TLR4 signaling in the urinary tract. Adapted from Ragnarsdóttir *et al.* [38].

Inflammatory mediators

Activation of the uroepithelia by UPEC triggers intracellular signaling followed by transcription and secretion of peptides. Transcriptomic analysis of mice bladder cells after UPEC inoculation showed induction of genes in the Tlr, Jak-Stat, T and B cell receptor signaling pathways [165]. Analysis of the mucosal transcriptomic response in humans is problematic due to the invasive procedures it requires. However, biopsies have demonstrated that uroepithelial cells produce IL-6, CXCL8 and a variety of other cytokines involved in UTI, depending on the disease state of the patient [166]. The proteins that are secreted into the bladder lumen reflect the activated signaling pathways [167].

Cytokines are defined as small molecules capable of mediating signaling between cells but several have multiple functions and may be further categorized as hormones, peptides or glycoproteins [168]. Members of the TNF family of cytokines are capable of inducing apoptosis in cells via activation of NF-κB, MAPK or death signaling. The interferon (IFN) family consists of type I (including IFNα/β) and type II (IFNγ) IFN's, and are involved in the antiviral immune response. Cytokines that have chemotactic ability are termed chemokines. Interleukins (ILs) are a group of multifunctional cytokines that play an important role in the immune response.

Chemokines

Chemokines are a group of small (8-14 kDa) cytokines that target specific cell populations. Chemokines bind to specific G protein binding receptors and thereby

attracting the cell to inflamed tissues by following the chemokine/cytokine gradient. Chemokines are divided into two major (CXC and CC) and two minor (C and CX_3C) subgroups based on the arrangement of cysteine residues [169].

CXC chemokines containing a glutamic acid-leucine-arginine (ELR) sequence near the N-terminus preceding the first cysteine residue are called ELR positive and are potent neutrophil chemoattractants and promoters of angiogenesis which bind the CXCR1 and CXCR2 receptors. CXCL8 is an ELR positive CXC chemokine, and was the first chemokine to be associated with UTI [170]. CXCL8 is produced in response to inflammatory stimuli such as IL-1, TNF-α, immune complexes and bacteria [171] and secreted by the mucosa during symptomatic [170] and asymptomatic [19] UTI. CXCL8 is essential for PMN recruitment to the bladder [172]. Other ELR positive CXC chemokines are CXCL1, CXCL2, CXCL3 (GRO-α, β and γ) and CXCL5 (ENA-78).

ELR negative CXC chemokines attract mononuclear cells, and the IFN inducible subset of these are inhibitors of angiogenesis and have antimicrobial activity [173]. These include CXCL9 (MIG), CXCL10 (IP-10) and CXCL11 (I-TAC).

CC chemokines target monocytes, dendritic cells and NK cells. Examples are CCL2 (MCP-1), which attracts macrophages, CCL5 (RANTES), which attracts T cells, mast cells, eosinophils and basophils and the eosinophil chemoattractant CCL11 (Eotaxin).

Interleukins

More than 40 cytokines are currently designated ILs [174]. They are divided into eight families depending on structure and function; 1) the IL-1 family, 2) the common γ-chain cytokine family, 3) IL-10 family, 4) IL-12 family, 5) Th2 cytokines, 6) ILs with chemokine activity, 7) IL-17 family and 8) other ILs.

Acute phase ILs in UTI include IL-1 and IL-6. IL-6 is an endogenous pyrogen which stimulates acute phase-reactant release from the liver and is detected in urine and serum of children with febrile UTI [21]. IL-1α and IL-1β are both proinflammatory, endogenous pyrogens, but IL-1β requires processing by caspase-1 for activation. The IL-1 receptor antagonist (IL-1RA) is released in response to the same stimuli that leads to IL-1 production [174]. IL-1 is detected in urine during cystitis [175].

ILs may also act as growth and proliferation factors, drive cell differentiation and have anti-inflammatory activity.

Polymorphonuclear neutrophil infiltration

Approximately 60% (>$3x10^6$ cells/ml blood) of circulating white blood cells are PMNs. PMNs are phagocytic granulocytes that respond fast to acute infection and migrate to the site of infection. During UTI, PMN migration follows an CXCL8 gradient that is secreted by the activated epithelia. Elevated levels of soluble CXCL8 in serum seem to inhibit neutrophil migration [176,177], and the CXCL8 gradient may be bound to the blood vessel epithelia. IL-8 binds the CXCR1 and CXCR2 receptors on the PMN and upregulates β_2 integins on the PMN surface. These bind to intracellular adhesion

molecules (ICAMs), expressed on the endothelial surface and facilitate extravasation of PMNs trough the endothelial wall and into the infected tissues.

Bacterial clearance in urinary tract is dependent on the CXCL8 receptor CXCR1 [178]. In mice, knock-out of the CXCL8 receptor homologue *mIL-8Rh* results in impaired PMN recruitment, kidney infection and subepithelial entrapment of PMNs [179], leading to tissue damage and renal scarring. CXCR1 was the first discovered UTI-susceptibility gene, and in APN-prone children CXCR1 expression lower than in controls [37].

Activated PMNs may kill bacteria though release of antibacterial granules, phagocytosis or formation of neutrophil extracellular traps (NETs). Phagocytosis may be mediated through antibodies and complement factors, and is suggested to take place in the subepithelia [180] rather than urine, where high levels of IL-8 may favour degranulation [181]. NETs form when activated PMNs release DNA covered in histones, enzymes and granular proteins into the environment. The NETs immobilize the bacteria and possibly kill them, but it is unclear if NETs are formed in urine during UTI [182].

Antimicrobial peptides

AMPs are expressed by neutrophils and epithelial cells in the urinary tract. AMPs are positively charged and contain 15-45 amino acids. The charged, hydrophilic domain of the AMP is drawn to negatively charged bacterial cell membranes thereby disrupting the membrane, resulting in bacterial cell lysis. AMPs in the urinary tract include α-defensins, β-defensins, cathelicidin (LL-37) and hepcidin. Cytokines like CXCL10 (also known as IP-10) and CXCL9 (also known as MIG) have antimicrobial activity, but their effect on UTI is not well documented. Lactoferrin and lipocalin show antimicrobial activity through sequestering of iron [183].

The complement system

The complement system consists of over 25 proteins and fragments that circulate in blood as inactive pre-cursors. The complement system may be activated via the classical, alternative or lectin pathway. All pathways require proteolytic cleaving of the components to generate active mediators. The complement system has four major functions:

1. Chemotaxis of macrophages and neutrophils (C5a)
2. Opsonization, which enhances phagocytosis of the antigen (C3b)
3. Lysis of cell membranes (C5b, C6, C7, C8, C9)
4. Clumping of antigen-covered cells (C3b, CR1)

The complement component C3 has been found in the urine of bacteriuric patients [184], but the *in vivo* effects are not clear. C3 may promote internalization of *E. coli* into mouse proximal tubular epithelial cells, and C3 knockout mice were resistant to kidney infection in an UTI model [185]. The internalization of UPEC was mannose-reversible and not detected when a *fim* negative strain was used, suggesting that the C3-promoted internalization is dependent of type 1 fimbriae [186].

Adaptive immunity

A number of immunodeficient mice breeds have been screened for UTI susceptibility [187]. *Severe combined immunodeficiency (Scid)* mice, which lack B and T cells, carry higher bacterial loads after challenge with UPEC after one week, compared to wild-type mice but T cell deficient nude mice responded similarly to controls [188], which suggests a role for the humoral response in UTI. Mice with *γδ T cell* or *Ifn-γ* deficiency have increased bacterial counts compared to wild-type mice [189]. Recently, γδ T cells, which are abundant in the gut mucosa, have been shown to play a pivotal role in murine UTI by being a source of IL-17A during acute UTI in a murine model [190]. IL-17A transcript levels peaked 48h after UPEC challenge in wt mice, and *Il-17a-/-* mice had significantly lower levels of PMN infiltration and inflammatory cytokines [190].

The role of adaptive immunity in UTI has not been fully clarified, but recent studies suggest that it may play part in certain aspects of the host response to UTI. Dendritic cells are recruited the bladder submucosa in murine models of UTI, but depletion of these does not impair bacterial clearance [191]. The bladder mucosa also contains lymphocytes which increase in number during bacteriuric conditions [192]. Patients with long-term bacteriuria may develop follicles in the bladder mucosa which contain lymphocytes, B cells and plasma cells and resolve spontaneously when the infection is cleared [192,193]. In addition, secretory Immunoglobulin (Ig)A and IgG is present in the urine of females and males with UTI, increase with ascending infection [194], and are capable of inhibiting UPEC adherence *in vitro* [195]. In some studies *in vivo* effects contradict this importance, as JHD B cell deficient mice were not susceptible to UTI [189], and a vaccine protecting against UTI did not elicit a humoral response [196].

Protection against recurrent UTI with the same UPEC strain has been demonstrated in a reinoculation model, as mice were resistant against UTI when inoculated with the same UPEC strain on a different occasion [197]. In the protected mice T cells were recruited to the bladder and specific IgG antibodies were present in the serum and urine. The protective response was transferrable via transfer of splenic T cells or serum from previously infected mice to uninfected mice [197]. T regulatory cells have so far not been found in the bladder mucosa.

Vaccine development against UTI has as yet not been successful, although several promising candidates are continuously emerging [198]. OM-89/Uro-vaxom® (OM Pharma, Myerlin, Switzerland) consists of a mixture of outer membrane proteins from 18 different bacterial strains and a metaanalysis [199] it reduces incidence of UTI with up to 30%.

Intramuscular injection of a vaccine based on the type 1 fimbrial adhesin FimH has been successful in mice and non-human primates [200], and a Phase II clinical trial of a vaginal vaccine consisting of 10 heat-killed uropathogens significantly reduced infection rates in women [196].

AIMS

The major aim of this thesis was to define asymptomatic bacteriuria from a molecular perspective. We used a human inoculation model for deliberate establishment of ABU.

In detail, the aims were to:

1. Define the host influence on bacterial adaptation during long term *E. coli* 83972 ABU
2. Define the effects of genetic variation on the proteomic host response during long term *E. coli* 83972 ABU
3. Define the mechanisms of *E. coli* 83972 induced cell signaling activation and transcription
4. Define the kinetics of the host transcriptomic signature during *E. coli* 83972 ABU and the effect of P and type 1 fimbriae on its dynamics

METHODS

The human inoculation protocol- a model for studies of the interaction between host and microbe during colonization of a sterile mucosa

In my thesis I have used data from two human colonization trials to define the host response to ABU and its effects during bladder colonization. The human ethics committee at Lund University approved the studies, and patients gave their informed consent.

Inoculation study I (Dnr LU 742/2001) Clinical Trial Registration RTP-A2003 (International Committee of Medical Journal Editors), clinicaltrials.com. This study was a placebo-controlled clinical trial aimed at determining the clinical effect of *E. coli* 83972 inoculations as a prophylactic against recurrent UTI. The clinical results are described in Sundéns 2010 paper [26]. Patients were inoculated with 30 ml of 10^5 cfu/ml *E. coli* 83972 on three consecutive days. Monthly urine samples and cultures were gathered for up to 12 months. Data from this study was used for *Paper I* and *Paper II*. The sampling and inoculation protocol is shown in Figure 4.

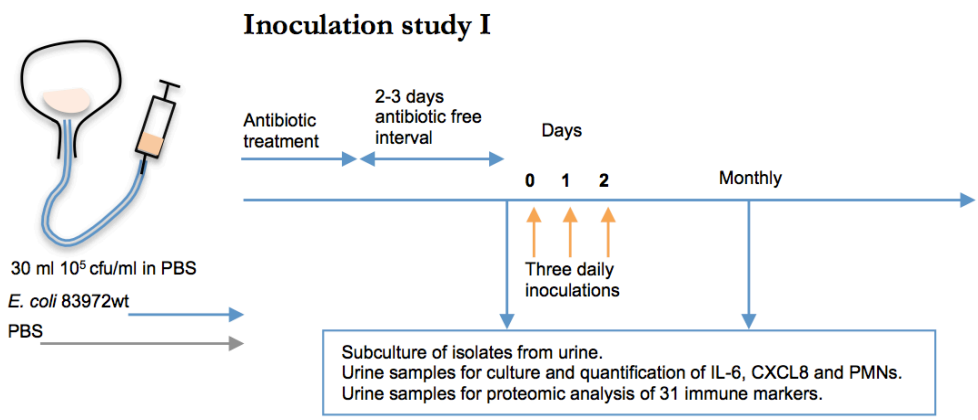

Figure 4. Inoculation study I. Inoculation and sampling protocol.

Inoculation study II (Dnr LU 298/2006; 463/2010). This study was a clinical trial aimed at determining the effect of *E. coli* 83972 inoculations on the host response kinetics and the *in vivo* transcriptome of *E. coli* 83972. Patients were inoculated with 30 ml of 10^5 cfu/ml of *E. coli* 83972, *E. coli* 83972*fim* and *E. coli* 83972*pap* on three separate occasions. Urine and peripheral blood samples were collected for up to 7 weeks. Data from this study was used for *Paper III* and *IV*. The sampling and inoculation protocol is shown in Figure 5.

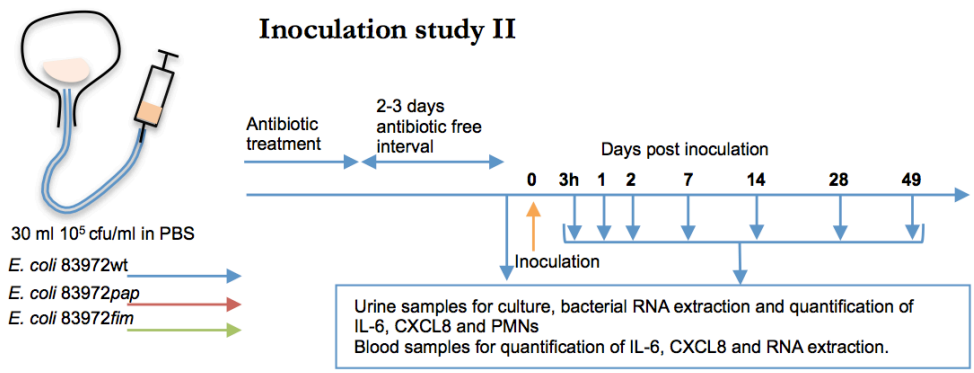

Figure 5. Inoculation study II. Inoculation and sampling protocol.

PRESENT INVESTIGATION

Paper I. Host imprints on bacterial genomes – rapid, divergent evolution in individual patients

The aim of this paper was to determine how individual hosts influence the genetic evolution of *E. coli* 83972 during asymptomatic carriage in the urinary tract.

Background

Bacterial evolution is fast and occurs through mechanisms like point mutation and recombination. The colonization of a host organism increases the selective pressure on the microbe to successfully adapt. Whether this increases the virulence of the strain or selects for commensalism has been debated. In the urinary tract, the loss of virulence may be beneficial. The microbe would avoid triggering detrimental host defenses and allow for monoclonal colonization of a nutrient-rich niche with little competition against other strains. The ABU prototype strain *E. coli* 83972 was isolated during a long-term study of ABU from a young girl, who carried the strain for at least three years without side effects and it carries defective *fim* and *pap* genes. Human inoculation with *E. coli* 83927 protects against symptomatic UTI, and allows for studies of adaptation of a single strain to different hosts.

Results

E. coli 83972 is related to UPEC strain CFT073 but has lost and gained genetic material

The complete genome sequence of *E. coli* 83972 (5,131,397-bp and a 1,565-bp cryptic plasmid) was solved using Sanger sequencing approach and deposited in the GenBank database. The genome was compared to one enterohemorragic *E. coli* strain (O157:H7 Sakai), one *E. coli* K12 strain (MG1655) and three UPEC isolates (CFT073, UTI89 and 536). The 83972 genome was most similar to CFT073, but had reduced genome size. Several genes associated with virulence were inactivated by deletions (*fim*), point mutations (*PapG* and an internal stop codon in *HlyA*) and phage insertions (*focD*,

rstB). Six prophages were unique in type of chromosomal location, and two of these were 83972-unique.

In vivo growth increases genome plasticity resulting in host specific genomic alterations

Sequential *E. coli* 83972 isolates were collected from patients participating in a clinical trial of prophylactic *E. coli* 83972 ABU. To distinguish the effects of host response-driven mutations from random events, the parent strain was also grown in pooled human urine for more than 2000 generations. Pulse field electrophoresis after treatment with restriction enzymes (*XbaI* and *Avr*II) found alterations in genome structure in 5/16 re-isolates, but none in three *in vitro* grown 83972 isolates. The changes in genome structure were consistent in different clones from each isolate, and were specific for each host and time point. Three re-isolates (PI-2, PII-4 and PIII-4) and one clone from the *in vitro* culture (4.9) were selected for sequencing. In total 37 loci were polymorphic to the parent strain, and 34 of these were found in the *in vivo* re-isolates (19, 9 and 6 in the respective hosts). The majority of the alterations were SNPs (n=29) in coding regions, and 27 substitutions were non-synonymous. To determine if the alterations were transient, re-isolates from a later time point were collected and subjected to single locus Sanger sequencing. In PII, 17/19 SNPs were still present after 126 additional days of colonization, and in PII 4/9 SNPs (*mdoH, rpiR, fecI, yejMI*) remained after 125 additional days of colonization. The patients had been inoculated with *E. coli* 83972 on a separate occasion. Several genes (*Fec1, BarA/UvrY*, *mdoH*, *frmR*) were repeatedly altered in isolates from separate inoculation periods, but did not appear altered in the *in vitro* grown isolates.

In vivo growth induces host specific transcriptomic, proteomic and phenotypic alterations

The effect of *in vivo* propagation on gene expression was determined by subculture of PI-2, PII-4, PIII-4 and 4.9 in pooled human urine, and further transcriptomic analysis of these isolates. *In vivo* growth increased the number of deregulated genes compared to the parent strain, was host specific host and matched by protein and phenotypic expression. Isolate PIII-4 had increased expression of genes affecting motility and FliC was the most up regulated protein on the bacterial surface. A phenotypic assay demonstrated increased motility in the isolate.

The mucosal host response to E. coli 83972 inoculation

The mucosal host response was analyzed in patient urine samples after *E. coli* 83972 inoculation. The host response to *E. coli* 83972 was highly variable in the three patients. PMN infiltration ($P<0.0001$) and CXCL8 concentration ($P<0.008$) was significantly higher in PI, who was the patient carrying the isolate with highest number (n=19) of mutations. This patient also expressed the highest levels of the IL-1 inhibitor IL-1RA. CXCL10/IP-10, CCL2/MCP-1, CXCL1/GROα, IL-1RA, and sIL-2Rα concentrations varied significantly between the three patients.

Conclusions

This paper demonstrates that *E. coli* 83972 evolves to adapt to the individual host during ABU, suggesting that the adaptation is actively driven by the human host. The adaptation to the individual host is reflected by a differential activation of the mucosal host response in the hosts. The results indicate that during ABU, the adaptation favors commensalism.

Paper II. Genetic control of the variable innate immune response to asymptomatic bacteriuria.

The aim of this paper was to characterize the human innate mucosal host response to the prototypic ABU strain *E. coli* 83972. Furthermore, the association with genetic polymorphisms in host response was investigated. Through this unique approach, we have excluded the bacterial strain variation accompanying natural infection.

Background

Variation in the mucosal host response has been noted in individuals with ABU, leading to uncertainty about the extent of innate immune reactivity during ABU and use of host response parameters as a basis for diagnostic and therapeutic decisions in clinical practice. Urothelial cells respond to infection by activating signaling pathways that result in secretion of cytokines. Compared to symptomatic UTI, the host response to ABU is low and does not cause systemic immune activation [21]. The host response to ABU reflects both the properties of the infecting strain and the host immune status, which makes it difficult to distinguish the level of host variation in the host response to ABU. In this study, host response parameters in 23 patients inoculated with the same *E. coli* strain were analyzed prior, during and after repeated inoculations. An extensive urine proteome profile was established. Through this unique approach, we have excluded the bacterial strain variation accompanying natural infection. The effect of genetic regulation on the magnitude of the host response was determined, as patients were genotyped for specific promoter polymorphisms in *IRF3* and *TLR4* that accumulate in ABU-prone individuals.

Results

Long-term (>3 months) *E. coli* 83972 ABU was established in 23 participants and PMN numbers, CXCL8 and IL-6 were quantified in 233 bacteriuric urine samples. PMN numbers and CXCL8 concentrations were significantly increased ($P<0.0001$) compared to sterile conditions, but IL-6 levels were not affected. The levels of CXCL8 and PMN infiltration varied between individuals, and the individual-specific level was identical upon reinfection at a separate occasion.

An extended screening of the urine proteome was performed on 87 samples from 11 patients. A cytokine/chemokine panel was used to detect 31 proteins involved in innate and adaptive immunity. In addition to CXCL8, we detected an increase in chemoattractants involved in neutrophil (CXCL1/GROα), mast cell or eosinophil (CCL5/RANTES, Eotaxin-1), monocyte (CCL2/MCP-1) and T cell (CCL5/RANTES, CXCL10/IP-10) chemotaxis. An increase in inflammatory regulators IL-1α and soluble

IL-1 receptor analogue were detected, as was the T lymphocyte/dendritic cell product soluble IL-2 Receptor α (sIL-2Rα). Nineteen proteins were not detected; the majority of these were involved in adaptive immunity.

The stable level of cytokine expression after repeated infection indicated a genetic background. Eleven patients were genotyped for ABU-associated *IRF3* and *TLR4* promoter polymorphisms. Three (V, VI, VII) of five *TLR4* genotypes associated with ABU were detected in five patients, which had lower neutrophil numbers (P=0.0014), IL-6 (P<0.0001), CXCL10/IP-10 (P<0.0001), CCL2/MCP-1 (P=0.008) and sIL-2Rα (P<0.0001) concentrations compared to the six remaining patients. Four patients carried the heterozygous (-925, -776, A/G-C/T) *IRF3* promoter genotype associated with ABU. These patients had lower neutrophil numbers (P=0.01) and concentrations of IL-6 (P=0.0007) and CCL2/MCP-1 (P=0.0001) than the remaining group.

Conclusion

This paper demonstrates that genetic polymorphisms in genes that regulate innate immunity have a significant impact on the magnitude of the host response during bacterial colonization of the urinary tract. The results also indicate that innate immune mediators drive the host-specific, low immune response to ABU.

Paper III. Bacterial control of host gene expression through RNA Polymerase II

The aim of this paper was to examine if ABU strains affect host gene expression and to determine the quality of the transcriptional host response to ABU.

Background

Individuals with ABU carry the same numbers of bacteria in the bladder as symptomatic patients, but activate a low host response. The failure of ABU strains to induce a destructive inflammatory response has been attributed to their lack of functional virulence factors, but the induction of the low host response that accompanies ABU has not been determined. It is thus unknown whether the ABU strain is actively affecting the host response during ABU. Bacterial transcriptional regulation during ABU *in vivo* has been characterized [201] but the quality of human host transcription during ABU has not been investigated. To determine the transcriptional host response during ABU, we characterized the transcriptional response to uroepithelial *E. coli* 83972 challenge *in vitro*, and the transcriptional profile of systemic leukocytes during *E. coli* 83972 ABU in human hosts.

Results

Inhibition of Pol II dependent host gene expression by E. coli 83972

Three patients were inoculated with *E. coli* 83972, and urine samples and peripheral blood samples were collected prior and up to four weeks after the inoculation. *E. coli* 83972 inoculation induced a transient but low increase in urinary PMN and CXCL8 concentrations after 24 hours, but the patients experienced no symptoms despite carrying bacterial concentrations of $>10^7$ cfu/ml. The 24 hour time point was chosen for whole genome transcriptomic analysis of peripheral blood leukocytes (PBLs) and compared to the sample taken prior to inoculation. Inoculation reduced the expression (unadjusted *P* values less than 0.05 and absolute $\log_2$ fold change greater than 0.5) of 63.3% of the probe sets. The majority of commonly regulated genes in all patients were downregulated (87.3%, 140/160 probe sets). The top category of commonly downregulated genes was transcriptional regulators. The list of commonly regulated genes was used to generate networks based on gene interaction. We identified a network of downregulated genes associated to RNA Polymerase II (RNA Pol II).

E. coli 83972 may downregulate RNA Pol II-dependent uroepithelial transcription

To further characterize the effect of bacterial colonization on uroepithelial gene expression, we used an *in vitro* model. Kidney epithelial cells (A498 cells) were grown in six-well plates and stimulated with the prototypic ABU isolate *E. coli* 83927 or the clinical APN isolate *E. coli* CFT073 (10^9 cfu/ml) for four hours. RNA was harvested and whole genome transcriptomic analysis was performed using non-stimulated A498 cells as controls. Genes with adjusted *P* values less than 0.05 and absolute $\log_2$ fold changes greater than 0.585 were further analyzed. Stimulation with the ABU strain regulated fewer genes than the APN strain (1288 vs. 1861). The majority of the regulated genes (1186) were similarly regulated, 669 were only regulated by APN and 96 were only regulated by ABU. Six genes were inversely regulated, and these and the 96 ABU-specific genes were defined as "ABU-specific" and further analyzed. Genes related to RNA Pol II transcription were the largest category (22.5%) of ABU-specific genes. Seven transcriptional activators involved innate immune regulation (*FOS*, *LBH*), growth (*CTNNB1*), proliferation (*OSR2*) or other functions were repressed. Three transcriptional repressors (*DACH1*, *BANP* and *DDX20*) were upregulated. DACH1 is a regulator of *FOS* transcription, and DDX20 enhances NF-κB suppression. Gene expression was validated by real-time polymerase chain reaction (RT-PCR) and Western Blotting.

Inhibition of RNA Polymerase II phosphorylation

Successful elongation of messenger RNA (mRNA) requires phosphorylation of Serine (Ser) 5 and Ser2 on the RNA Pol II C terminal domain (CTD), which may be detected by immune-peroxidase staining using anti-phospho Ser2 antibodies and used as a measure of active transcription. Four hours of stimulation with the ABU strain reduced RNA Pol II phosphorylation in kidney (A498), bladder (J82, HTB-9) and primary tubular epithelial cells, compared to medium. The APN strain did not reduce RNA Pol II phosphorylation. The ability to suppress RNA Pol II phosphorylation was examined in the same epidemiologically defined collection of isolates that *E. coli* 83972 was found in. Clinical ABU isolates had a significantly ($P<0.001$) higher capability of suppressing RNA Pol II phosphorylation compared to APN strains. Interestingly, so did fecal strains. *E. coli* 83972 also inhibited RNA Pol II phosphorylation in two colon cancer cell lines (HT-29 and DLD-1).

Supressed transcription of genes involved in innate immune signalling

The TLR4 is essential for the host response to UTI, and low TLR4 expression has been linked to susceptibility to ABU [45]. The destructive inflammation that accompanies APN is heightened in *Irf3* and *Ifnb*-negative mice [41], indicating a protective role for the type 1 interferon response during acute kidney infection. The TLR4 and the type 1 Interferon pathway were suppressed in systemic leukocyte RNA from inoculated patients. The suppression was mainly focused on intracellular signaling cascades and transcription factors (*STAT1*, *PLCG1*, *CREB1*, *FOS*, *JUN* and *SOCS1* were suppressed in all patients).

In A498 cells, the TLR4 and the type 1 Interferon pathway genes were less activated after stimulation with the ABU strain compared to the APN strain, and phosphorylation arrays verified the decreased activation of these genes.

Conclusion

This paper demonstrates that ABU is an active rather than passive process, and that ABU and fecal strains decrease RNA Pol II phosphorylation in host epithelial cells, resulting in suppressed transcription and pathogen-specific innate immune pathways.

Paper IV. Conversion from ABU to virulence: Effects of P and Type 1 fimbriae on human gene expression, signal transduction and symptoms

This study uses a transcriptomic approach to determine the kinetics of the local and systemic host response during ABU, and how this response is altered by type 1 and P fimbrial adherence to the uroepithelial mucosa.

Background

Symptomatic UTI causes acute inflammation in the lower and upper urinary tract, but individuals with ABU do not have increased pyrogen concentrations and the host response is low. In the urinary tract, *E. coli* resist urine flow by expressing P fimbriae or type 1 fimbriae, which adhere to specific receptors [72] and activate different pathways [41]. The mucosal activation results in urine PMN infiltration and release of cytokines and AMPs, but the mechanisms that regulate the magnitude of the inflammatory response are unknown. P fimbriae are present in the majority of APN isolates and approximately 30% of cystitis isolates, and type 1 fimbriae are expressed by virtually all *E. coli* strains. In an attempt to extend the inoculation therapy to patients without residual urine, P or type 1 fimbrial expression was restored in *E. coli* 83972 by introduction of functional copies of *papG* or *fim* genes into the chromosome. Previous inoculation studies have been performed using *E. coli* 83972 transformants carrying plasmids with the *pap* or *fim* gene cluster [92-94,98]. The P fimbriated variants established faster in the urinary tract [92] and induced a higher local host response without causing symptoms [98], but the type 1 fimbriated variants did not adhere to bladder cells, affect the host response or improve bacterial establishment [94]. This study addressed if the re-acquisition of functional P or type 1 fimbriae alters the host response on a transcriptomic and proteomic level in a human host.

Results

Fimbrial expression in vivo induces a distinct mucosal host response

Five patients (P I-V) were inoculated with the parent strain *E. coli* 83972wt and on later occasions with *E. coli* 83972*fim* and/or *pap*. The host response to bacterial inoculation and prolonged bacterial carriage was examined in a total of 13 colonization periods. Urine and peripheral blood samples were collected prior inoculation and after three hours and one, two, seven and 14 days after inoculation. Type 1 fimbrial expression was gradually downregulated from +++ to + after one week, but *E. coli* 83972*pap*

reisolates expressed functional fimbriae throughout the colonization period (two weeks). Urine PMN infiltration, CXCL8 and IL-6 reached peak levels in each individual during the first 48 h after *E. coli* 83972 wt and *fim* inoculation, but the peak levels after *E. coli* 83972*pap* inoculation occurred after one week or later. *E. coli* 83972*pap* induced a statistically significant increase (P<0.03) in urine IL-6, compared to *E. coli* 83972*fim.*

Early reprogramming of host gene expression

We expected the host response to combine genes regulated by *E. coli* 83972wt with genes specifically regulated by the fimbriae. Inoculation with *E. coli* 83972*pap*, *E. coli* 83972*fim* and *E. coli* 83972wt induced a distinct host response with small overlaps with the wt strain and a majority of genes being specific for the strain 3 hours after inoculation and at peak time-points of gene regulation.

The regulated genes were further characterized by examining the top five gene networks induced 3 hours after inoculation with *E. coli* 83972*fim* in P I and *E. coli* 83972*pap* in P V. Central genes in both samples included *NFKB*, *VEGF*, *ERK1/2* and *HISTH4*. These were surrounded by activated genes in the *E. coli* 83972*pap*- induced network (106/173), and suppressed genes in the *E. coli* 83972*fim*-network (108/175). An upstream regulator analysis predicted MYC and IL-15 to be inhibited 3 hours after *E. coli* 83972*fim* inoculation in P I. The analysis could not predict an upstreams regulator based on the rregulated genes 3 hours after *E. coli* 83972*pap* inoculation in P V.

Adhesion to the mucosa influences the early host response kinetics in systemic peripheral leucocytes

To study the effects of fimbriae on the host, RNA was obtained from PBLs after inoculation with *E. coli* 83972*fim* and *pap* and compared to *E. coli* 83972wt. Differentially expressed genes (fold change ≥ 2.0) were defined intra-individually using the pre-inoculative blood sample corresponding to each colonization period as a background. The kinetics of gene regulation after *E. coli* 83972wt inoculation was highly variable between the patients. In contrast, *E. coli* 83972*fim* induced peak gene deregulation the first 48 hours in all four patients and *E. coli* 83972*pap* induced peak gene deregulation at 48h or later in all four patients. Differentially expressed genes (fold change ≥ 2.0) were further analyzed using the Ingenuity Pathway Analysis software.

The peak response to E. coli 83972 fim includes the NK cell and Integrin signalling pathways

To characterize gene expression induced by inoculation of *E. coli* 83972*fim,* we first examined the early sample (3 hours) collected from the high responder P I. NK cell signaling and Integrin signaling were amongst the top five regulated pathways. Several genes involved in NK cell signaling were persistently suppressed compared to after wt inoculation at three (P=0.001), 24 (P=0.04) and 48h but returned to basal levels after one week. This pattern was identical in P IV (24h P=0.03, 48h P=0.0008). The top

downregulated genes (FC <-1.41 3-48h in P I and P IV) were killer cell lectin-like receptors and adaptive immunity regulators. The innate immunity inhibitor leukocyte immunoglobulin-like receptor (LILRB1) was upregulated in P I-IV. Genes involved in Integrin signaling were mainly upregulated in P I.

The peak response to E. coli 83972 pap includes the Interferon and Pattern Recognition Receptor signalling pathway

To characterize gene expression induced by *E. coli* 83972*pap* inoculation, we first examined the sample collected 36 h after P Vs symptomatic episode. P V developed APN 17 days after inoculation with *E. coli* 83972*pap*, received immediate antibiotic treatment and recovered fully. IFN signaling and PRR signaling were identified as the top-scoring canonical pathways. IFN signaling was significantly upregulated (P<0.0001-0.02) in all patients compared to the wt inoculation. The upregulation occurred one to two weeks after *E. coli* 83972*pap* inoculation. Activated transcription factors were *IRF1*, *IRF9*, signal transducers and activators of transcription 1 (*STAT1*) and 2 (*STAT2*). The PRR pathway includes TLR4/TRIF dependent signaling, which is activated by P fimbriated *E. coli* [41]. PRRs involved in nucleic acid (*TLR7* and 8), flagella (*TLR5*) and intracellular recognition (*RIG1*, *NOD2*) were upregulated in PV. PPR signaling was significantly upregulated in P IV-V (P<0.0001) one or two weeks after *E. coli* 83972*fim* inoculation compared to after wt inoculation.

Conclusion

This paper demonstrates that P fimbriae are key factors for activation of disease related, pathogen-specific signaling pathways that cause symptomatic infection.

DISCUSSION

We have evolved a host response that rejects or accepts the microbes that colonize our bodies using the most intricate system of antibodies, immune cells and receptors. Simultaneously, microbes have evolved strategies to persist in their host. The study of molecular interactions between host and microbe has grown into a thriving field over the past decade, and by defining the molecular process during host-microbe interactions that do not cause disease, we are provided with tools to diagnose the infections that do. Our results add to this field by providing direct molecular evidence of host specific evolution of bacterial genomes, and characterizing the host proteome and transcriptome during ABU.

Microbial adaptation

We have evolved a host response that rejects or accepts the microbes that colonize our bodies using the most intricate system of antibodies, immune cells and receptors. Simultaneously, microbes have evolved strategies to persist in their host. ABU strains often carry inactivated or truncated virulence genes, and can therefore persist for long periods without activating a host response. Sequencing of the prototypic ABU isolate *E. coli* 83972 demonstrated that it is closely related to the APN isolate *E. coli* CFT073, but has acquired mutations in several virulence factors. During colonization of the human urinary tract, 83972 continued to acquire mutations or deletions that affected the gene expression, protein expression and phenotype of the isolate. In PII a shift to a less immunogenic iron scavenger occurred during *in vivo* colonization. Modifications of virulence factors have been documented in sequential isolates from patients with chronic *Heliobacter pylori* infections [202,203] and recurrent *E. coli* bacteraemia [204]. The host-specific adaptation strategy has since been demonstrated in cystic fibrosis isolates from a long-term study in Denmark [205]. The doubling time of 83972 during in vitro growth in human urine ranges from 45 minutes to 60 minutes [87], but whether this rate is stable during *in vivo* long-term ABU is unknown. The bladder is a protected but dynamic niche, and it is likely that the strain may increase or decrease its replication rate depending on the host environment. Acute cystitis isolates express high levels of ribosomal genes, indicating that symptomatic UTI is accompanied by active bacterial replication [206], but so far the bacterial gene expression profile during ABU has not been determined. In our study, the subcultured patient isolates showed a highly differential

expression of genes mainly involved in iron acquisition, stress response, motility and amino acid metabolism, which corresponded with the protein synthesis and phenotype of the isolate.

The understanding of microbial *in vivo* evolution is extremely important for the understanding host-microbial relationships. It clarifies not only the level of selective pressure during persisting infections, but also which genes are required for effective bacterial persistence. In the future, it may inform us about the individual host immune status, rendering a commensal to be as informative of the host it has colonized, as it is about its own species.

The innate immune function is influenced by the host genetic profile

The host-specific adaptation of 83972 correlated with a highly variable host environment. Innate immune parameters and PMN infiltration in urine were significantly different between the three patients in *Paper I*. After examining the microbial aspect of long-term ABU, we proceeded to characterize the host response.

During long-term bacteriuria, 83972 elicits low but significant mucosal activation. In total, we detected 13 proteins that are secreted by the asymptomatic uroepithelia, and 18 that were undetected in both sterile urine and bacteriuric samples. The majority of the detected proteins were involved in chemotaxis. The majority of the undetected proteins were associated with adaptive immunity, indicating that the low host response during ABU is mainly result of innate immune activation. The levels of the host response were identical during repeated colonization periods in the same patient.

In our patient population, the carriage of ABU-related promoter polymorphisms predicted a decreased level of host response during ABU. Our group has previously reported that promoter polymorphisms in *TLR4* [40] and *IRF3* [41] are highly prevalent in individuals prone to ABU. The functional effect of these on the host response was determined in *Paper II*, demonstrating that patients with ABU-associated *TLR4* polymorphisms, of which several result in a decrease in *TLR4* expression, had lower expression of chemokines and cytokines regulated by both the MyD88-dependent and TRIF/TRAM-dependent TLR4 pathways. The low-responder genotype also exhibited decreased urine concentration of the IL-2Rα chain. Soluble IL-2Rα is secreted by activated B cells, monocytes, eosinophil granulocytes, dendritic and NK cells [207,208]. The decrease in sIL-2Rα may indicate that TLR4-signaling has a positive effect on the activation of adaptive immune cells or NK cells. It has long been known that lymphocytes infiltrate the bladder mucosa during long-term bacteriuria [193], and NK cell activation has been reported to facilitate *E. coli* clearance [209]. The lower amounts of sIL-2Rα could potentially reflect a lower activation state of NK cells in ABU prone patients, theoretically facilitating continued colonization of the urinary tract.

IRF3 is normally expressed at high constitutive levels in most cells, and acts with IRF7 to initiate type I IFN gene transcription. The two ABU-associated *IRF3* SNPs were

first discovered in a screening for candidate genes in SLE patients [47]. However, IRF3 expression has been implicated in a variety of bacterial infections. In the urinary tract P fimbriated *E. coli* activate IRF3, and *Irf3*-/- mice are susceptible to kidney infection [41]. IRF3 also plays an amplifying role in the inflammatory response by macrophages to the periodontal pathogen *Porphyromonas gingivalis* [210], limit bacterial growth *in vitro* and enhance macrophage and neutrophil phagocytosis of *Yersinia pestis* [211]. Studies in astrocytes have suggested that IRF3 may suppress neuroinflammation through regulating proinflammatory miRNA miR-155 that may be induced by both cytokines and TLR ligands [212].

Patients with ABU-related *IRF3* SNPs exhibit increased promoter activity [41], and had lower levels of IL-6, CCL2 and PMN infiltration. While IL-6 is downstreams of IRF3, CCL2 is induced upon NF-κB activation but also by IL-6 signaling [213]. IL-6 is an endogenous pyrogen that has both pro-inflammatory and anti-inflammatory effects [213], while CCL2 is a monocyte and macrophage chemoattractant that has been detected in several populations with UTI [214,215], and high levels have been connected with APN [214] and correlated to kidney disease [216]. The heterozygous *IRF3* genotype may protect the host by decreasing more specific aspects of the inflammatory response during bacteriuria. It is unclear in what stage the protective response is activated, and further studies would be necessary to determine the functional aspect on how *IRF3* gene products protect against kidney infection.

Suppression of host gene expression

The moderate host response during ABU is far from the destructive inflammation that accompanies APN. It is activated by several UPEC virulence factors that have been epidemiologically and clinically characterized and are often not expressed by ABU isolates. Our group has previously described how P and type 1 fimbriated *E. coli* activate specific innate immune pathways [41,156], but the specifics of how 83972 moderates the host response during ABU are not completely understood.

In *Paper III*, we propose a new mechanism by which ABU strains may persist in the urinary tract. Stimulation of uroepithelial kidney, bladder and colon cancer cell lines with 83972 inhibited Ser2 phosphorylation of the RNA Pol II CTD. Though an epidemiologic analysis we found that this was a common trait among the majority of the examined ABU and fecal *E. coli* isolates. Inhibition of Ser2 phosphorylation locks RNA Pol II in an elongation block, which results in a lower level of transcription in the cells [217]. By comparing the transcriptome of 83972-stimulated to CFT073-stimulated A498 cells, we could pin-point 112 genes that were specifically regulated by only 83972. The majority of the genes were involved in suppression of transcriptional regulation. This indicates that 83972 downregulates certain aspects of transcription. PBLs from patients that had been inoculated with 83972 downregulated the majority of commonly regulated probe-sets (87.5%), of which the major category was transcriptional regulators. This

indicates that during ABU, leukocyte chemotaxis during requires downregulation of factors that are expressed when the patient is not bacteriuric. Genes involved in the TLR4 and IFN pathways were also downregulated in PBLs, which may reflect a shift in priorities from detection of pathogens to phagocytic activities.

Inhibition of an inflammatory response by targeting the transcriptional machinery has recently been investigated for therapeutic purposes. Ser2 phosphorylation is regulated by the recruitment of positive transcription elongation factor (P-TEFb) by bromodomain protein 4 (BRD4), or independently by BRD4 [218]. Pretreatment of macrophages with a BRD4 inhibitor suppressed the LPS gene response upon subsequent challenge [219]. In our model, a cochallenge of A498 cells with 83972 and CFT073 inhibited the Ser2 phosphorylation which ordinarily accompanies CFT073 stimulation. BRD4 has recently been identified as a therapeutic target in acute myeloid leukemia, multiple myeloma, Burkitt's lymphoma, NUT midline carcinoma, colon cancer, and inflammatory disease. Histone de-acetylase (HDAC) inhibitors increase the susceptibility to bacterial and fungal infections but protect against septic shock [220].

The recent discoveries suggest that modulation of basal mechanisms of transcription may be a promising field for development of antimicrobial therapies. Our study adds that commensal strains may use this mechanism to induce an early RNA Pol II arrest, halt the transcription of innate immune mediators and thus establish in the urinary tract.

The effect of P and type 1 fimbriae for the mucosal host response

The long, symptomfree periods during *E. coli* 83972 ABU are an important improvement for patients with recurrent UTI. Attempts to increase the colonization ability of 83972 to patients without residual urine have used plasmid transformants of 83972 expressing the *prs*, *pap* or *fim* gene cluster [24,92-94,98], but these lost the fimbrial phenotype within 72 hours after inoculation. Therefore, the chromosomal *E. coli* 83972*pap* and *fim* variants were created. The patient group was selected after the criteria from the previous studies to compare the effect of the chromosomal location on colonization ability and host response induction.

Host response parameters peaked 24-48 hours after inoculation with 83972wt and 83972*fim*, confirming the earlier observation that type 1 fimbriae do not promote inflammation in the human urinary tract [94]. The peak in PMN infiltration coincided with an increase or peak in the number of deregulated genes in PBLs, suggesting that ongoing pyuria may be detected in a blood sample.

The type 1 fimbriated transformant, 83972*fim* gradually lost its fimbrial phenotype within one week after inoculation in all patients. ABU strains accumulate mutations in the *fim* gene cluster [79], and fresh isolates often express type 1 fimbriae only after subculture [70,206].

The P fimbriated transformant, 83972*pap*, retained its phenotype. Inoculation with P fimbriated *E. coli* 83972 induced a strikingly distinctive host response, with a delayed

peak of urine IL-6, CXCL8 and PMN infiltration up until one to two weeks. In previous studies with plasmid 83972*pap*, the elevation of host response parameters occurred during the first 48 hours after inoculation and the patients remained asymptomatic. Few studies have been able to determine how long after establishment of bacteriuria the onset of symptoms occurs, and a recent study of events preceding symptomatic UTI, found that the prevalence of pre-clinical UTI (defined as the prevalence of symptoms, pyuria and bacteriuria the days prior to report to the clinic) was increased in strains that were negative for the *pap* gene cluster and none of the 14 *pap*-positive isolates had caused preclinical UTI [221].

The delayed host response after 83972*pap* inoculation may have several causes. The *pap* gene cluster is often associated to the *HlyA* gene, which is mutated in 83972. The avirulent 83972 background may thus be the cause of the delay. A second possibility is that the natural course of UTI may be longer than expected, or that P fimbriae have an immunomodulatory function.

Trancriptional signatures of type 1 and P fimbriated *E. coli*

Several pathogen-specific transcriptional signatures have been identified in peripheral blood leukocytes (PBLs) from patients with infections [222-225], and their accuracy have been reported to be as high as 85% [225]. Previous studies have shown that P fimbriated and type 1 fimbriated UPEC activate separate signaling pathways in uroepithelial cells [41,156], and human colonization studies have demonstrated that these activate a different mucosal host response [93,98]. The mucosal host response to UTI has been extensively studied over the years, but the host transcriptome during ABU has, to our knowledge, never been examined.

Gene regulation after inoculation with *E. coli* 83972*fim* was increased during the 48 hours that the strain expressed the phenotype, while gene regulation after *E. coli* 83972*pap* inoculation dramatically increased three hours after inoculation in PV and after one week in the remaining patients. It is known that intravenous of CXCL8 impairs neutrophil chemotaxis [19]. Three hours after inoculation with *E. coli* 83972wt *CXCL8* was downregulated in all five patients and remained suppressed at all analyzed time-points in *Paper IV* (up to two weeks, data not shown), indicating that *E. coli* 83972 inoculation may be detected in PBLs after only three hours.

The top regulated pathways after 83972*fim* inoculation were NK cell signaling and Integrin signaling, and NK cell signaling remained persistently downregulated in PI and PIV during the entire period that the type 1 fimbrial phenotype was detected. Upregulation of integrin signaling in PI may reflect a rapid chemotactic response, preparing for tissue adhesion and extravasation.

To understand the symptomatic event in PV, we analyzed the blood sample that was taken 36 hours after the onset of symptoms. The top-scoring pathway was IFN signaling, which has previously been connected to the inflammatory response during APN [41].

Examining the samples preceding the symptomatic event in PV we saw a low but marked upregulation of several genes that gradually increased up to the onset of symptoms. A significant increase in IFN pathway genes was found in all patients one week after *E. coli* 83972*pap* inoculation. In both PII and PV, IRF9 was upregulated in the sample preceding the symptoms. IRF9 mediates type 1 IFN signaling by associating with STAT2 and STAT1 to form the interferon-stimulated gene factor 3 (ISGF3) complex, which has been previously associated with the antiviral host response [226]. Future studies might determine whether IRF9 may have a predictive value for the outcome of an infection.

The second top regulated pathway after *E. coli* 83972*pap* inoculation was the PRR signaling pathway. Several intracellular PRRs involved in recognition of viral RNA, bacterial cell wall components or flagella/flagellin were upregulated. *E. coli* 83972 has the capability to express flagella, and a patient isolate of the parent strain in *Study I* had increased expression of the *fliC* gene, protein and exhibited increased motility. It is possible that the addition of P fimbrial binding results in increased flagellar potential for virulence.

Concluding remarks and future prospects

The objective of this thesis was to clarify how *E. coli* 83972 prevails in the bladder without causing a detrimental inflammatory response. We have demonstrated three mechanisms that we believe aid in this; (1) the loss of virulence of the ABU strain during colonization of different hosts (2) the presence in the host carriage of genetic polymorphisms that lower the host resistance to ABU and (3) the active suppression of host transcription by ABU strains, including pathways required for an efficient antibacterial defense. In addition, we consider it interesting that the presence of ABU or UTI may be detected in host PBLs, and suggest this has potential for development of a non-invasive method of detecting, understanding and monitoring the course of UTI or ABU. To further develop this project, the kinetics of PBL transcription after *E. coli* 83972 inoculations should be investigated and furthermore, the bacterial factor that allows 83972 to suppress host gene transcription.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Mer än hälften av alla kvinnor har haft en urinvägsinfektion. Infektionen uppkommer när bakterier når urinblåsan och börjar växa. Immunförsvaret känner igen bakterierna, reagerar med en inflammation och symptom som sveda, trängningar och smärtor i nedre magtrakten uppstår. Om bakterierna klättrar upp till njurarna sprider sig inflammationen dit och orsakar ryggsmärtor och feber.

Återkommande UVI är ett stort problem. Den enda fungerande behandlingen mot UVI är antibiotika och dessa patienter utvecklar ofta allergier mot vissa sorter. Samtidigt ökar antibiotikaresistensen snabbt bland bakterier som orsakar UVI. Många experter varnar för att vi inom snar framtid måste anpassa oss till att antibiotika helt enkelt inte går att använda vid många och vanliga infektioner, däribland urinvägsinfektioner. Det finns således ett stort och brådskande behov av att utveckla och forska kring alternativ till antibiotika. För att kunna göra detta måste vi förstå infektionernas sjukdomsmekanismer in i minsta detalj.

Redan på 1950-talet fann man att individer kunde bära på stora mängder bakterier i urinblåsan utan att märka av det, så kallad Asymptomatisk bakterieuri (ABU). Senare studier visade att ABU är vanligt, ofarligt och till och med skyddar mot urinvägsinfektioner. Bakterier som orsakar ABU skiljer sig från de som orsakar sjukdom, och saknar många av de gener som vi vet är grundläggande för att orsaka inflammationer i urinvägarna. På grund av den skyddande effekten av ABU utvecklades ett protokoll för kolonisering av urinblåsan med en viss ABU-bakterie som döpts till *Escherichia coli* 83972. Denna har identifierats som en icke sjukdomsframkallande (”snäll”) *E. coli* bakterie, och har burits av patienter i mer än tre år utan att orsaka symtom. Kliniska studier i Sverige och USA har visat att kolonisering med *E. coli* 83972 skyddar patienter mot urinvägsinfektioner. Tyvärr begränsas behandlingen av att *E. coli* 83972 endast kan orsaka långtidskolonisering hos patienter med störd blåsfunktion som lämnar kvar resturin efter blåstömning. För att lösa detta problem krävs ytterligare forskning som kartlägger hur *E. coli* 83972 samspelar med värden och hur den skulle kunna förbättras i sin förmåga att kolonisera urinvägarna även hos patienter med normal blåsfunktion.

I vår studie har vi att kartlagt bakteriens DNA efter att den levt i olika patienter, och sett att den anpassar sig till den individ som bär den. Många av förändringarna i bakterien var av den typen att de gjorde den mindre benägen att orsaka en inflammation. Detta

stödjer vår hypotes om att vid ABU blir bakterien allt mer harmlös för den mänskliga värden.

Bakterien anpassar sig till sin miljö och därför undersökte vi vad immunförsvaret skickar ut i urinblåsan när det kommer i kontakt med *E. coli* 83972. Vi analyserade urinprover från 23 patienter under tiden de bar på bakterien. Vi fann 13 av de 31 protein vår utrustning kunde detektera, varav de flesta var associerade till det medfödda immunförsvaret. Vår grupp har tidigare definierat två gener, *TLR4* och *IRF3*, som har en speciell sekvens i patienter med ABU. Elva av våra patienter undersöktes för dessa sekvenser och vi fann att den sekvensen resulterade i betydligt lägre koncentrationer av protein samt antal vita blodkroppar i urinen.

För att avgöra hur immunförsvaret reagerar när en bär på *E. coli* 83972 undersökte vi blodprover från tre av våra patienter. Vi fann att de hade nedreglerat produktionen av mRNA som är ett förstadium till färdiga proteiner. Nedregleringen påverkade kända signalvägar som används av sjukdomsframkallande bakterier och även gener som är förknippade med generell mRNA- produktion. Vi såg liknande resultat när vi stimulerade *E. coli* 83972 på njurceller i labbet. Njurcellerna stoppade även aktiveringen av RNA Polymeras II som ansvarar för produktionen av mRNA. Detta visade för första gången att vi kan detektera symptomfri bakterieuri (ABU) med ett enkelt blodprov.

Vi gick vidare med kartläggningen av immunförsvaret genom att följa fem patienter som bar på *E. coli* 83972 med urinprover och blodprover. Patienterna fick även två modifierade *E. coli* 83972 bakterier, som tidigare studier visat är bättre på att hålla sig kvar i urinblåsan. Urinproven visade att de första två dagarna efter inokuleringen så aktiveras ett lågt immunförsvar som attraherar vita blodkroppar, men detta nedregleras efter en vecka. Den ena modifierade bakterien gav en oväntad förhöjning av immunsvaret. För att förstå varför den orsakade symptom så analyserade vi den totala mängden mRNA i blodproven. I alla fyra patienterna, oavsett förekomsten av symptom, så hade den bakterie som bar på P fimbrien uppreglerat en signalväg som vi tidigare kopplat till sjukdomsframkallande bakterier. Här föreslår vi att framtida analyser kanske kan detektera vilken typ av gener en bakterie bär på och hur länge den har varit närvarande i individen, genom analys av ett blodprov.

Sammanfattningsvis har vi funnit tre skäl till att bakterier som orsakar ABU inte orsakar inflammation och symptom. Bakterien anpassar sig för att inte orsaka inflammationen, individen som har ABU har genotyper som gör minskar deras immunsvar mot bakterien och att bakterien aktivt stänger ner immunförsvaret hos patienten. Dessa resultat är viktiga för att bättre förstå ABU och för att kunna utveckla nya alternativa behandlingar mot UVI. Resultaten pekar mot ett för varje patient unikt samspel mellan värd och bakterie. Förhoppningsvis ska vi genom dessa insikter i framtiden kunna erbjuda fler patientgrupper *E. coli* 83972 inokulering, kanske genom att molekylärt ”skräddarsy” *E. coli* 83972 till att exakt passa patientens unika egenskaper.

ACKNOWLEDGEMENTS

This thesis is built on the generosity of individuals who took time out of their private lives to participate in a clinical trial. None of this would have been written if it weren´t for you. I think that you participated because you believed in the project. So do I. There weren´t many of you, and I am glad to say that we met many times over these years. The days when you came into the lab were always the best ones. I really appreciated meeting you, talking and hearing about how you were doing. I hope you are all are well, and I wish you all the best. **Thank you!**

Björn Wullt, my supervisor. Thank you for believing in me, making everything sound easy and always being positive. Your passion for all things science have inspired me so many times over these years. Thank you for always listening to my ideas with an open mind and honest enthusiasm. It has truly been a privilege to work with you.

Catharina Svanborg, for being much more than an assistant supervisor and giving me the opportunity to work in your lab.

Bryndis Ragnarsdottir, my first supervisor and eternal roommate. Many a cat-pictures have been posted on our walls and many a "funny" joke has been shared. You taught me how to build primers, grow cells, run the flow, and thousands of other things. You even proofread my thesis! Thank you.

Ulrich Dobrindt and Jarek Zdziarski, for their scientific accomplishments that have inspired so much of what I have done. I have valued our conversations during these years.

I have been supported by many competent people during these years: Ann-Sofie Ling, who took the blood samples and met with patients, Annika Andreasson at the Immunology department, Kristina Andersson, who let me use their Luminex system and Jill Storry and Gabriella Godaly for the smiles and small-talks.

I have been lucky to have many fantastic co-workers both in and outside the lab. Sonja who has one of the biggest hearts, Maria who showed me how to work the microscope (thank you!), Natalia for scientific support and friendship, Majlis who taught me about "fosterutdrivningskaffe", Petter, Anki, Susanne, Manoj, Anna, Aftab, Bela and Karoly all spiced up my working days.

Lotta Glans. What can I say. You are an important part of this thesis. Thank you for so many things, among them leading the way. Emma Von Karis, Hanna Sundberg, Emmanuel Nyberg and Moa Andersdotter. The Bruna Djur gang. Thanks for being there- I´m back now!

Lisa Zetterberg, who kept me in shape by forcing me to lift weights twice a week during the extremely hot summer when I wrote this. YG 4-ever.

My team, Crime City Rollers, Malmö Rollerdeby. I love the lot of you, you fantastic human beings.

My family: Mamma, who since I was born believed that I can success in anything, and always told me that I am equal to anyone on this earth. Jag älskar dej mamma! Pappa, porque me simpre has enseñado ser una buena persona, y lo importante que es siempre respetar a otros humanos, animales y la vida. Te quiero. My beloved sisters Katarina and Alexandra, who I wish I could spend more time with! You are both stars.

Best things last: Henrik Möller, my life partner. So many advice you have given to me that just turned out to be infinite truths. Coming home to you each day is the best thing in the world. I love you.

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