From the Department of Neuroscience
Karolinska Institutet
Stockholm, Sweden

Tissue responses and host transcriptomics
in bacterial infections

Jorrit Boekel

Stockholm 2011
Abstract

Bacterial infections can damage host tissue and are as such a potential threat to their hosts. To protect themselves from pathogens, hosts therefore can employ diverse immune reactions. When bacteria are recognized by their hosts, complex signaling cascades are triggered that lead to an influx of specialized immune cells into the infected tissue and a change in tissue integrity. The inflammation that is mounted may eliminate the pathogens, but will also cause substantial tissue damage. The foundation for the inflammatory process is laid early, in the first 12 hours of infection. This thesis aims to reveal host responses within this early time frame.

While in vitro studies can yield highly detailed data on subjects as protein-protein and cell-bacterial interactions, they cannot reproduce all aspects that occur in a live animal, such as immune infiltration, nerve, and hormone effects. We have developed a kidney infection model of bacterial infection to study early whole-host responses to bacteria. Using micropuncture techniques, we delivered bacteria to a known nephron, from where the infection progressed. Within hours, we observed numerous physiological changes of the tissue volume bordering the infection. Infection kinetics could be visualized and showed markedly faster host responses to haemolysin (Hly)-carrying bacteria compared to Hly-knockouts. Tissue oxygen levels decreased in response to infection, possibly caused both by blood flow restriction combined with epithelial oxygen consumption. Blood flow shutdown at the infected nephron was due to activation of the coagulation cascade. Coagulation also protected against sepsis, as animals died due to bacteremia when this cascade was inhibited. Some of these phenomena could be found in the host transcriptome. We also found that a core of common gene expression exists in live host innate immune responses by applying bioinformatic methods on the gene expression measurements. This core had a strong IFN-γ signature, a cytokine which we consequently found increased in the blood stream, and expressed by cells in the spleen. We go on to show that IFN-γ downregulates transcription of several neutrophil-attracting chemokines, and that this does not occur through canonical effectors of either IFN-γ or inflammatory signaling pathways.

The data I present here show that using a live host infection model can reveal host processes that cannot be found using in vitro models. By combining this model with analysis methods that yield detailed data, early responses could be studied. The data show the importance of live models for discovering unknown contributors and functions in inflammation, which may lead to possible future medicine development.
List of publications

This thesis is based on the following publications, which are referred to in the text by their Roman numerals:


IV Boekel J., Frisan T., Rhen M., Richter-Dahlfors A. Inhibitory effects of IFN-gamma on bacterially-induced transcription in epithelium. *Manuscript*
# Contents

## Introduction
- The body and the pathogen ........................................... 1
- Infections of the kidney .................................................. 2
- Bacterial recognition: raising the alarm ............................... 4
- The call upon immune cells ............................................. 6
- Transcriptional control of the tissue response ....................... 9
- Comprehensive as in data and as in models .......................... 11

## Aims

## Results and Discussion
- An intravital model to study early-stage infections ............... 13
- Infections cause local blood flow and oxygen level alterations . 15
- Coagulation is a host response that protects against sepsis ... 16
- Gene expression studies of early host responses ................. 17
- IFN-γ is an early responding immune modulator ................. 19
- IFN-γ downregulates epithelial chemokine responses .......... 21

## Conclusions

## Perspectives

## My thesis for the layman

## Acknowledgements

## Bibliography
List of abbreviations

AP-1 activating protein 1
CCL chemokine (C-C motif) ligand
CCR chemokine (C-C motif) receptor
CD cluster of differentiation, compact disc
ChIP chromatin immune precipitation
CXCL chemokine (C-X-C motif) ligand
DAMP damage-associated molecular pattern
DC dendritic cell
f-MLP N-formyl-methionyl-leucyl-phenylalanine
GM-CSF granulocyte macrophage colony-stimulating factor
Hly α-haemolysin
ICAM intercellular adhesion molecule
IFN interferon
IL interleukin
iNOS inducible nitric oxide synthase
JNK c-Jun N-terminal kinase
LPS lipopolysaccharide
M-CSF macrophage-colony stimulating factor
NF-κB nuclear factor kappa-light-chain-enhancer of activated B cells
NLR Nod-like receptor
NKT natural killer T
PAMP pathogen-associated molecular pattern
Pol II polymerase II
PRG primary response gene
RNS reactive nitrogen species
ROS reactive oxygen species
SRG secondary response gene
TF tissue factor
Th T helper
TLR Toll-like receptor
TNF tumor necrosis factor
UTI urinary tract infection
Introduction

The body and the pathogen

No matter what its function or location, healthy tissue is the subject of homeostasis. Cells in the tissue continually strive towards a certain set point in their environment, whether it be salt concentrations or amount of cells in the tissue. When pathogens such as bacteria enter the tissue microenvironment, the risk exists that their presence will prevent the environment from upholding its set points, e.g. by consuming nutrients or damaging cells by toxins. The tissue can only endure a limited amount of imbalances like these, and changes too far from healthy homeostasis will eventually lead to compromised tissue and thus organ function, which is potentially dangerous to the organism. The entrance of the pathogen thus creates the need for the tissue to use drastic measures in order to safeguard its own state and that of the organism. From an evolutionary point of view, it has been stated that hosts need a vigilant immune response to prevent takeover of the soma by genomes that replicate much faster than that encoded in the germ line [1].

To eliminate pathogens, mammalian hosts possess both pathogen-specific adaptive immunity, which needs several days to be mounted, as well as immediate but less-specific innate immunity. Innate and adaptive immunity are intricately linked, and control each other’s behaviour. Thus, early events immunity are crucial for later responses. For these to occur, the pathogen must first be detected and identified. This is not as straightforward as it may seem, since in the bacterial kingdom only there are many different pathogens, most of which are closely related to commensal bacteria. The differentiation of pathogens from other bacteria is the subject of an entire field, and I will not go into details, but pathogens can carry identification molecules different from commensals, and the tissue damage that some pathogens induce is also a signal for the host. The tissue next has to use its weapons to defend itself while waiting for back-up. Cells in the tissue can produce substances such as antimicrobial peptides, that have a bactericidal effect. Then, the tissue will signal the surroundings that pathogens have been identified. This will lead to recruitment and infiltration of immune cells from the bloodstream. These specialized cells are made by the body to fight off pathogens, and will as such have a larger capacity to do so than the infected tissue whose main function can be something completely different. In healthy organisms there is a large supply of immune cells that can be used towards dealing with an infection.

The process of recruiting cells and the vascular response are termed inflammation. The four cardinal signs of inflammation, rubor, tumor, calor, dolor [2] (and the fifth, functio laesa), can be observed when an inflammation occurs at the surface of the body. And however an inflammation of an internal organ does not necessarily result in the full set, a number of cell communication events will have to take place before the described state of inflammation has
been reached. The symptoms of inflammation, which originated as a clinical term, appear when the inflammatory response recruits immune cells to the site of infection. Inflammation is seen as a part of the innate immune response, but can also be elicited in sterile tissue, for example in response to damage [3]. Not all components of the innate immune response however fall under the inflammation denomination. When I mention inflammations or inflammatory responses in this thesis, I talk about the ones resulting from a bacterial infection. I will limit the discussion to acute inflammation as opposed to chronic inflammation, and will talk about early host responses, defined as faster than 12 h post infection. Let us know take a more detailed look on the components of the innate immune response and their interactions.

**Infections of the kidney**

The kidneys are responsible for the production of urine from blood. To do so, each human kidney consists of >1 million nephrons, the functional units of the kidney (Fig. 1). The main part of a nephron is an epithelial tube through which primary urine, filtered from the blood by the glomerulus, flows. The epithelial cells then reabsorb sugars, ions, water, and amino acids by both active absorption and passive transport, in order to keep the organism in homeostasis. Reabsorbed substances are taken up by the extensive capillary network that surrounds each nephron, as the kidneys together receive ~20% of cardiac output in resting state. The resulting urine is collected downstream from the nephrons and is ultimately transported by the ureters to the bladder [4]. The supply of blood is critical for kidney function, as a steep decrease of it and the consequent renal ischemia lead to organ injuries. Aside from well-described morphological changes [5, 6, 7], renal ischemia induces an inflammation that contributes to the resulting tissue damage. These inflammations are characterized by an infiltrate of immune cells consisting of T and B cells, macrophages and neutrophils, of which T cells seem to be especially important in the early events [8].

Urinary tract infections (UTI) can be caused by a range of pathogens, which are assumed to enter the urinary tract from a colonic reservoir. When bacteria reach the bladder, they can there cause asymptomatic bacteriuria or symptomatic cystitis. Pyelonephritis ensues when bacteria subsequently manage to migrate to the kidney [9]. While UTI can be caused by *Proteus mirabilis, Klebsiella pneumoniae, Pseudomonas aeruginosa*, and other bacteria [10], the far largest share of infections is caused by *Escherichia coli* [11], to which I will limit discussions.

Uropathogens can express a range of virulence factors to enable colonization of their infection niche, which is partly modified by attachment to host cells. Type I fimbriae are an important virulence factor in bladder infections. These structures on the bacterial outer membrane bind the uroplakins lining the bladder wall [12, 13, 14], thus presumably protecting the bacteria from being flushed out. These fimbriae also allow the bacteria to enter bladder cells [15], making the bladder a possible reservoir for recurrent infections since
most antibiotic treatments do not affect intracellular bacteria [16]. From the bladder, bacteria migrate up to the kidney, which in mice requires a bacterial flagella [17]. Here, they may attach using P fimbriae which can bind certain glycolipids on renal epithelium [18, 19]. P fimbriae are considered to be important for kidney infections in different, but not all organisms [20, 21, 22, 23], but this may also hold true for type I fimbriae [24]. Recently, type I fimbriae were shown to be necessary for colonizing the center of the nephron in rats, possibly via inter-bacterial binding [25].

Other relevant bacterial virulence factors are protein toxins such as α-haemolysin (Hly), a toxin with dual activity which can lyse host cells at high concentrations [26], but also induce a number of signaling events in host cells at lower concentrations [27, 28]. These events can then lead to transcription of proinflammatory mediators. Hly, encoded by ~50% of uropathogenic E. coli [29], can be hypothesized to function to facilitate spreading, as it can induce effects in epithelial cells, and as a bacterial defense mechanism. Last but not least, bacterial outer membrane contains lipopolysaccharide (LPS), which not only serves as a protective shell, but also induces a strong immune response [30].

Using the bladder as a reservoir, bacteria can potentially ascend to the kidney multiple times to infect different parts of the kidney. Since this organ is so compartmentalized, kidney infections and accompanying host responses will be very local phenomena, unlike in the bladder, where bacteria can potentially cover large parts of epithelial surface. The renal structural characteristics also make it possible for the immune response to cause some collateral tissue damage when eliminating the bacteria, without losing total kidney function. Although this works when few nephrons are infected, collateral damage to many nephrons is potentially dangerous as larger parts of the kidneys are affected. Since the kidney has such a high degree of vascularization, the blood compartment is always close to where the bacterial infection is, making
translocation into the bloodstream a possible outcome in kidney infections. Indeed, gram-negative sepsis often originates from the genitourinary tract [31], and many E. coli strains that caused bacteremia carry P-fimbriae [32].

The kidney is a beneficial infection model, partly because of close proximity of the vascular system to the tubules. This eases study of tubulo-vascular interactions and lumen-to-bloodstream signaling events. Since the route to kidney infection naturally is via the bladder, experimental models can apply a similar bacterial route, termed ascending or retrograde infection. After bacterial delivery to the bladder, an incubation period in the order of days is used before kidney infection is normally studied. Among the number of significant advantages of this method are ease of use, high degree of similarity to natural infections, and feasibility for semi high-throughput experiments. However, when studying early infections, timing and mapping of bacterial arrival to a particular part of the kidney are crucial, and unattainable using a retrograde infection model. A different problem in infection biology is formed by the complications that arise when using live models. Cell cultures and cell-free systems can easily be studied in real time, which adds significant quality to time series analyses. In live models, the complexity of the hosts do not easily permit real time analysis of cellular-resolution processes, which would give infection researchers access to an extra dimension. Altogether, an infection model that addresses these issues would enable high-resolution analysis of early kidney infection.

Bacterial recognition: raising the alarm

When pathogenic bacteria enter the tissue, they have already conquered the defense system that is made up by physical barriers, such as the skin, mucus, beating cilia, and fluid flow. The body must now identify the bacteria, and it can do so in multiple ways. In a number of tissues, such as skin, there will be specialized immune cells that deal with the protection of the tissue [33]. They have receptors, both on their surface and intracellularly, that recognize specific bacterial molecules, termed pathogen-associated molecular patterns (PAMPs) [34]. These receptors activate cell mechanisms that prompt the cell to alert its surroundings, and will set off the inflammatory cascade. Even faster is the response of these cells to immediately start secreting antimicrobial products in an effort to kill the bacteria [35].

One of the well-known PAMP receptors that plays a major role in recognizing our model pathogen E. coli, is Toll-like receptor (TLR) 4, that acts as a receptor for LPS [36]. This receptor belongs to the TLR-family [30], which was first discovered as a homolog to Drosophila melanogaster protein Toll [37, 38, 39]. Although multiple PAMP receptors may be involved, TLR4 is assumed to play a major role in the renal host response to E. coli, as mice with a deficient receptor suffer higher bacterial loads in the kidney and reduced neutrophil infiltration [40]. As the kidney is lined with epithelium, bacteria will first be encountered by epithelial structures in the urinary tract. Though renal epithelium is not a specialized immune tissue, it is capable of mounting an
immune response. Differences have been found for different urinary tract epithelia in TLR4 expression and function: renal epithelium reacts in a much milder fashion towards LPS than bladder epithelium does, correlating with lower levels of TLR4 mRNA [41] in proximal tubular epithelium. However, another study found that TLR4 is expressed throughout the human urinary tract [42], and TLR4 has been found in rats to be upregulated in kidney tubules upon inflammation [43]. In cultured cells, P-fimbriated E. coli can upregulate TLR4 surface expression [44]. Other PAMP receptors play roles in the renal host response as well: the flagellin receptor TLR5 [45] has also been shown to be important [46], as has TLR2, which has been linked to a higher urinary tract disease incidence, be it against Gram-positive pathogens [47]. Tsuboi et al. show expression and reactivity of TLR2 in mouse tubular epithelium, but not for TLR5 [48]. However, Thr-5 knockout mice showed higher susceptibility for E. coli UTI [46]. Other members of the PAMP-receptor family include the Nod-like receptors (NLRs) [49], which recognize intracellular PAMPs, amongst which constituents of bacterial cell walls. In epithelial cells, it is not fully known if and how NLRs play a role in the detection of extracellular bacteria, since PAMPs may need to be translocated to the cytosol for NLR recognition. The possibilities may exist though, when one thinks of intracellular uropathogens in the bladder [50], endocytotic properties of kidney epithelium [51], uptake of bacterial products by epithelial cells [52], and the shown possibility of these cells to recognize bacterial products via the Nod1 protein [53]. It will be interesting to see whether more PAMP receptors will be discovered the coming years, and more specifically, what their nature is. It should be noted that apart from pathogen recognition, a number of PAMP receptors are also able to activate downstream responses by responding to endogenous ligands, such as to ceramide [44], necrotic cell lysates [54], or endogenous proteins [55, 56, 57]. However, some claim that in a number of cases, contamination of preparations of these endogenous agonists with PAMPs are to be blamed for these responses [58].

After bacterial recognition, PAMP receptors can activate several signaling pathways that induce immune responses in the epithelium. TLR4 and other TLRs can activate the transcription factor NF-κB via MyD88. This adaptor protein recruits IRAK kinases, which then leads to degradation of the IκB subunit, setting NF-κB free for translocation to the nucleus [59]. Although this protein is central to inflammatory responses, there are a number of other transcription factors that are activated via PAMP receptors, including the activating protein (AP)-1 protein family (via p38 and JNK) and IRF3 (via TRIF), depending on which cell type reacts to the stimulus. Certain NLRs can also activate NF-κB, but several are known to act in a protein complex termed inflammasome [60]. This complex, containing amongst others caspase 1 and an NLR, can cleave pro-forms of proinflammatory cytokines such as interleukin (IL)-1β, thereby enabling their secretion. Inflammasomes can be activated by a variety of signals thought to be sensed by NLRs, amongst which extracellular ATP, bacterial toxins or insoluble crystals like asbestos. Common for these is that they all cause generation of reactive oxygen species
(ROS) and cellular K\(^+\) efflux [60]. These signals are thought to mediate information about cellular damage such as that caused by an infection and have been named damage-associated molecular patterns (DAMP) [61]. PAMP and DAMP sensors are likely to collaborate in mounting a proper immune response to a pathogen. As a result, epithelial cells will start producing cytokines upon recognition of bacteria, including proinflammatory IL-6, IL-1β, and tumor necrosis factor (TNF) [62], which alert the surrounding cells.

The epithelium is not the sole immunocompetent cell type in the kidney though (Fig. 2). As already noted, cells that act as sentinels are dispersed throughout tissues. In the kidney, few lymphocytes and macrophages are found [63], but a network of dendritic cells (DC) is found in the interstitium [63, 64]. It remains a question what the function of these cells is in the early host response in the kidney. Unlike in the intestinal tract, where DCs extend into the intestinal lumen [65] and are able to take up pathogens transepithelially [66], kidney DCs could not be detected to stretch into the urinary space [64]. They may have a different role than first samplers of pathogens though, as infections can induce fast epithelial breakdown [67, 68], thereby possibly freeing the way to DCs for pathogen components. DAMPs and cytokines produced by epithelium and other cells at the infected site would also reach the DC network. Although highly probable, it is not well known whether kidney DCs play a role in the innate immune responses in this organ, but they are well capable of presenting antigens in renal lymph nodes [69].

**The call upon immune cells**

The inflammation process is characterized by recruitment of different functional populations of leukocytes to the inflamed site. As different mature leukocytes are in the bloodstream at the same time, there is a need for a communication system via chemokines and other substances to recruit the right cells with the specific functions necessary to combat the infection. Chemokines produced by kidney epithelium are mainly of the neutrophil attracting kind, such as chemokine (C-X-C motif) ligand 1 (CXCL1), CXCL2, and IL-8/CXCL8 [41, 62, 42]. The receptors of these Glu-Leu-Arg-motif positive chemokines have been shown to play an important role in urinary tract infection susceptibility [70, 71]. Monocyte attracting chemokines have also been reported to be produced by epithelial cells [62]. Many reports give different results on which of these proteins are produced by renal epithelium, which may be attributed to the experimenters’ different cell lines, primary cell-preparations, stimulus substance, and bacterial strains. The fact remains that epithelial cells are capable of starting an immune response, probably attracting a mixed population of immune cells.

The basally secreted cytokines and chemokines will diffuse and reach the endothelium, which carries receptors for these proteins (Fig. 2). Proinflammatory cytokines will activate the endothelium, thereby enabling it to dock immune cells via special receptors. This classically happens by presenting intercellular adhesion molecules (ICAM) such as ICAM-1 to leukocytes that
Figure 2: Schematic depiction (cross-section) of cell recruitment to a tubular infection. Epithelial cells react to bacteria, activating the tissue, which recruits leukocytes. Resident DC and fibroblast are shown in the interstitial space.

Roll on endothelial selectins with their selectin ligands [72]. Selectins are expressed upon endothelial activation by fusion of selectin-containing Weibel-Palade bodies with the cytoplasmic membrane [73]. The leukocytes will bind ICAM-1 through their integrins (e.g. LFA-1) and are then docked. The necessary step in between rolling and docking is leukocyte activation, which can be done by chemokines presented on the endothelium. This stimulates the cell to change conformation of its integrins [74, 75], allowing ICAM-1 binding. Chemokines can be produced by the endothelial cells themselves, but can also have an epithelial origin since they can be transported via transcytosis [76]. It has been questioned if the sequence of events is indeed the one described, since inhibiting rolling does not always inhibit adhesion, unless rolling is inhibited by >90% [77]. Lastly, these endothelial pathways differ somewhat per organ and blood vessel type; recruiting leukocytes is a process that is carefully regulated.

Neutrophils or polymorphonuclear granulocytes are considered the cannon fodder of the immune system and believed to have a life span of ~8 h. This view has recently been questioned using in vivo labeling methods showing circulating neutrophil half-lives of 90 h [78]. They can be rapidly recruited to an inflammation, where they can deploy an arsenal of weapons to attack the bacteria. Phagocytosis, production of reactive oxygen and nitrogen species [79], and production of hypochlorous acid [80] are all powerful bactericidal mechanisms. Once neutrophils have been recruited to an inflammation, they are considered to have no way back into a naïve state, and although their life span is enhanced by proinflammatory mediators [81], cell death can ensue after phagocytosis, resolving of the inflammation, anti-inflammatory cytokines, and other stimuli. Activation starts when the cells are recruited and encompasses large changes in their cytosolic contents: neutrophils contain a number of different granules of which the contents can be released extracellularly or
lysosomally in a controlled fashion [35]. Upon phagocytosis, fMLP or TNF stimulation, neutrophils can deploy a respiratory burst [82], resulting amongst others in superoxide production by NADPH oxidase, which could theoretically kill bacteria. It is likely but not certain whether superoxide actively reacts with bacterial components, as its reactivity is pH-dependent [83]. Another neutrophil defense system I would like to address here is the reactive nitrogen species (RNS) production system. RNS are made by the enzyme inducible nitric oxide synthase (iNOS), expressed upon neutrophil contact with diverse proinflammatory signals [84, 85]. The resulting RNS damage bacteria in a number of ways, amongst which are interfering with the bacterial respiratory chain [86] and inhibiting DNA replication [87], but also synergizing with ROS, resulting in even more toxic products [88]. Recently, neutrophil extracellular traps (NET) have been discovered to kill bacteria in vitro, and were shown present in mice infection models [89]. In vivo killing and implications for bacterial infections in humans have however not been shown for NETs yet. Other than being equipped with bactericidal mechanisms, neutrophils produce chemokines and cytokines themselves [90], thereby controlling inflammation. This has been a topic of some debate, and it was recently shown that using a very pure population of neutrophils resulted in a low amount of cytokines and chemokines, while a high amount of the anti-inflammatory cytokine IL-10 was produced [91]. Although it does not describe kinetics or in vivo situations, the paper points out that neutrophils may well have a controlling, downregulating role in infection, and are not just the simple cannon fodder I referred to at the start of this paragraph.

Other blood-borne cells that infected epithelium can recruit are monocytes, which come in subsets with distinct adhesion molecules and chemokine receptors. These cells represent a smaller blood population of leukocytes than neutrophils do (5-10% vs. 40-60% in humans). Monocytes can, as opposed to neutrophils, be viewed as not fully mature, as they not only are able to produce cytokines and perform phagocytosis, but also can differentiate into macrophages and DCs [92]. The subset dubbed inflammatory monocytes can be recruited to an inflammation via chemokine receptors such as chemokine (C-C motif) receptor 2 (CCR2), which depends in part on neutrophils that secrete monocyte attracting substances [93]. Although this paradigm divides the immune response into distinct neutrophil and monocyte stages, epithelium also can produce monocyte-attracting chemokines directly. Some investigators have argued for an independence of recruitment on neutrophils [94]. The second subset of monocytes, sometimes called resident or non-classical monocytes, is a somewhat more elusive set. It was shown in mice that this subset displayed patrolling behaviour, crawling over the endothelium, and were amongst the first cells to respond to an infection [95]. It is unclear if monocytes can develop into DCs and macrophages very fast, and they may initially function as inflammation controllers. Both macrophages and DCs may of course also be useful after the early innate immune response. They will be able to resolve the inflammation, phagocytose and clear debris, kill remaining pathogens, and act as antigen-presenting cells to build up adaptive
Although lymphoid cells are for a large part grouped under adaptive immunity, there are a number of them that do play roles in innate immunity, two of which I will cover here. These cells are found in lymphoid organs and in low percentages in peripheral blood, but some also populate mucosal tissues as resident lymphocytes. The kidney is as far as we know not populated by lymphocytes. Blood lymphocytes can typically be recruited to inflammations via receptors CXCR3 and CCR5 [96, 97, 98]. Natural killer T (NKT) cells are cells that possess characteristics (i.e. surface markers) of both natural killer cells and T cells, hence the name. Their best known subset, invariant NKT (iNKT) cells are directly reactive towards certain bacterial glycolipids via their invariant T cell receptor [99]. This receptor is also able to recognize host-derived antigens (also glycolipids) presented to them by other cells, especially when in combination with a cytokine stimulus [100]. The hallmark responses from these cells are secretion of interferon (IFN)-γ and IL-4, but also GM-CSF [100]. NKT responses are said to be almost immediate, which would fit well with a role in early host responses, and NKT cells have indeed been shown to amplify the immune response to LPS through IFN-γ production [101]. Another lymphoid cell involved in innate immunity is the γδ T cell. These cells differ from T lymphocytes in that they have a different and invariant T cell receptor. Strongly reactive to the bacterial metabolite (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate [102], they can drive inflammation via cytokine and chemokine production [103, 104, 105, 106]. The mechanism through which γδ T cells produce IL-17 has recently been shown to be a direct reaction to IL-23 and IL-1 [107]. The division of T lymphocytes into a large number of different subsets is based mainly on functional aspects and surface markers. Every now and then, a new subset is found within an old one, with a new function. This means that there may be more functional classes of lymphocytes waiting to be discovered. The future will show if there are more cells to be cast for new roles in innate immunity.

Transcriptional control of the tissue response

The described inflammatory process is subject to a large amount of control, in order to avoid excess collateral damage. Part of this control is exerted at the level of transcription, one of the main driving mechanisms of inflammation. I have already highlighted a number of signaling routes that converge on transcription, and these differ somewhat per cell type, inducing in each cell the appropriate behaviour. Before discussing transcription more in depth, one has to remember that mRNA and protein levels are not always correlated [108], as not only transcription is a regulated process, but so is the translation of the resulting mRNAs.

Transcription is often considered a binary process, e.g. either NF-κB is in the nucleus inducing transcription of proinflammatory genes, or it is not. The multistep process that mRNA expression is poses though a number of opportunities for regulation. First transcription has to occur, for which RNA
polymerase II must bind the chromatin via a series of transcription factor binding events, chromatin state alterations and cofactor recruitments (Fig. 3) [109]. Transcriptional programs elicited by proinflammatory agents are often divided into primary and secondary response genes (PRG and SRG, respectively) by their production’s speed and dependence on newly synthesized proteins. It has been shown in macrophages that those PRGs that responded fast to an inflammatory stimulus (LPS) did not require nucleosome remodeling to clear the chromatin [110]. They were instead found to have Pol II pre-assembled to their promoters, generating low levels of unspliced transcripts [111]. Activating macrophages with LPS increased transcription rates and enabled transcript maturation of the PRGs, without the prior need to clear the chromatin and assemble a transcription complex. This finding indicates strong linkage between transcription and splicing machinery. Another paper described how TLR-signaling induced the synthesis of protein ATF3. This protein subsequently inhibited transcription through what seemed to be chromatin modification, thereby limiting transcript production of genes induced via TLR4 [112].

Full-length pre-mRNA can be altered by splicing [113, 114]; where the average gene size is 27 kb, that of the average mRNA is only 1.5 kb [115]. An example of inflammatory mRNA regulation through splicing can be seen in how the adapter protein MyD88, critical for LPS and IL-1 induced host responses through NF-κB, is spliced in response to LPS, generating an isoform termed MyD88s [116]. Since this isoform lacks the ability to phosphorylate the next protein in the NF-κB cascade, it behaves as a dominant-negative inhibitor to LPS and IL-1 signaling. Alternative splicing can thus be a mechanism to temper immune responses.

Degradation is the third major influence on gene expression that I would like to discuss here. In itself a regulating mechanism, coupling a high transcription rate to efficient degradation enables fast adjustment to a changing environment. For example, mammalian short-lived transcripts have a half-life of about 15 min [117] and genes of the same functional classes are often degraded equally fast [118]. The protein Zc3h12a is an RNase inducible via...
TLRs and seems to degrade a set of mRNAs to control an inflammatory response [119]. The TTP protein binds to AU-rich elements in mRNAs, leading to removal of the poly-A tail and increased turnover of TNF mRNA [120]. Not only directed regulation via synthesis of inhibitors occurs: certain mRNAs have a sequence-encoded degradation rate. This hard-wired property is partly responsible for the three waves of gene activation by TNF. It has been found that TNF-induced mRNAs can be divided in three groups based on their 3'-untranslated regions, each with a different half-life [121]. Numerous other mechanisms of mRNA control exist, and the sheer multitude of them indicates the importance of regulating the inflammatory response, but also its great complexity and versatility.

**Comprehensive as in data and as in infection models**

Genome-wide transcriptional responses to bacterial pathogens have been extensively studied in *in vitro* models. Among the advantages of these studies are cell type specificity of the measurements, the controlled environment, and high-resolution data. An array of bioinformatic methods is available to analyze the large amount of data (routinely 30,000 data points per experiment) resulting from such experiments. Using functional classifications like Gene Ontology [122], can clarify data, while time-series cluster analysis can predict gene functions from kinetic data [112].

Live animal models do not possess the same resolution, but have advantages over cell cultures in that signaling will be between all relevant cell types involved, including possible distant signaling effects from other organs [123, 124]. For example, the kidney signals to a number of other organs in inflammatory conditions [124]. Burn injuries are also considered inflammatory sites, and prime the systemic immune system’s TLR responses in later responses [125]. Live models can thus be advantageous for study of whole-animal responses to infection.
Aims
This thesis is based on four papers, which are thematically related to one another by their investigation of early innate immune responses. The general aim was to discover unknown host responses to bacterial infections. More specifically, the aims of the papers were:

- to establish a method to analyze bacterial infections and whole-animal responses in real time.

- to elucidate the role of altered tissue homeostasis as an early host response to bacteria.

- to deduce biological knowledge from whole-genome mRNA level information in a complex host response.
Results and Discussion

An intravital model to study early-stage infections

The problem that sparked the making of Paper I was that there was no real-time based method to address certain questions confined to early time points in infections in live animals. Although one can use e.g. luminescence imaging to do so, the resolution and sensitivity of this method are relatively low compared to widely available techniques for in vitro studies. Two-photon microscopy was an interesting candidate technique, because it provides a resolution almost as high as for confocal microscopy, coupled to the possibility of imaging in live animals. The technique is based on a pulsed infrared laser that is led through an objective lens, resulting in crowding of photons of relatively low energy. Excitation of fluorophores only happens when these are simultaneously hit by two photons, due to the photons' low energy. Even with photon crowding, this event is so unusual that it only happens in the femtoliter sample volume that is the focal plane. Consequently, photobleaching outside that volume is avoided [126]. This principle also enables restriction of tissue damage, and the long wavelength of infrared light makes imaging deep into the tissue possible [126]. The technique has successfully been applied to kidney studies, where endogenous autofluorescence of proximal tubules can be used for structural identification [127, 128].

To be able to track and visualize the bacteria in the kidney, we engineered the uropathogenic E. coli strain CFT073 [129] to express a single genomic copy of the gfp+ gene by placing this gene under control of a constitutively active $P_{L\text{tetO-1}}$ promoter. We discovered that in vivo expression from this promoter was more stable than from the previously used promoter that drives ribosomal gene rpsM (unpublished). Four day retrograde infections of rats caused large abscesses on the renal cortex, and multiphoton microscopy showed a disappearance of autofluorescent tubules and blood flow from necrotic areas, indicating

Figure 4: Schematic of the method used to locally infect rat kidneys, image the infection in the live rat using 2-photon microscopy, and isolate the infected tissue for expression analysis (described later) by taking a biopsy.
a loss of tissue integrity. The ascending model was thus unconducive to the study of starting and early infections. We therefore developed a method to deliver bacteria directly to the proximal tubules. This was a considerable step towards analyzing early kidney infections, as we gained spatial and temporal control of the infection. We exposed the kidney via subcostal flank incision, keeping it fully functional throughout the procedure, and injected bacteria into the nephron (at 40 nl/min) using glass capillary pipettes [128, 130] (Fig. 4). Much faster injections would cause tissue damage, and we typically injected each site for 10 min, which equals a delivery of approximately $5 \times 10^5$ bacteria per nephron. A 10 kDa dextran-coupled cascade blue dye was co-injected with the bacteria, which allowed for injected tubule identification as the dextran was endocytosed by proximal tubule cells. To locate the infection by eye, nearby tubules were injected with black mineral oil. Shortly after injection, few bacteria were found in the nephron, presumably because most bacteria did not bind the tubular wall and were flushed out. Subsequent bacterial multiplication showed a large increase of the remaining bacteria that filled the tubule within 5-8 h of infection. Immune cell recruitment also became apparent within this time frame. Almost 24 hours after injection, the bacteria had been eradicated from the tissue, and the only bacteria that could be found seemed to have been phagocytosed. A large amount of leukocytes had been recruited to the infection by this timepoint and tissue integrity was lost.

The method we have developed facilitates the study of early events in the infection process, and showed drastic tissue responses within an 8 h time frame. The compartmentalization of the kidney confines the infection and the immune response to a small focus at the superficial renal cortex, which has a double advantage. Imaging is made easier, since we know where to look, and an exact timing is possible, eliminating the need to worry about time-heterogeneous responses. This as opposed to bladder infections, which can be more heterogeneous due to their larger “infection space”, and kidney infections caused by retrograde infection methods, as outlined earlier.

This method could potentially be used to analyze the effects of bacterial virulence factors on infection and host response kinetics in vivo. We chose to study the effect of Hly, since it is carried by ~50% of uropathogenic E. coli isolates. Isogenic hlyA knockout bacteria elicited slower blood flow responses than bacteria harbouring the toxin. Our group has previously shown that Hly can induce intracellular $\text{Ca}^{2+}$-oscillations in epithelial cells, leading to transcription of inflammatory cytokines [27]. This signaling mechanism may be a possible explanation for the faster induction of the immune response. Hly has dual activity, and the necrosis that may result from its pore-forming activity at higher concentrations may also hasten immune responses, as necrosis is a potent inflammatory signal [131, 132]. There has also been a recent report of Hly causing loss of cellular $\text{K}^+$ that induces p38 phosphorylation [28], which can activate immune responses. A similar $\text{K}^+$ flux would induce inflamma-some activation as mentioned earlier. Whatever the mechanism may be, it is
clear that Hly has an immuno-modulatory effect on the tissue response.

Infections cause local blood flow and oxygen level alterations

Using the described infection model, we found that bacterial infection induced a previously unknown restriction of blood flow to infected tubules, which prompted us to delve deeper into this host response. In perI we subject the vascular response found in Paper I to a closer examination. We find that between three and five hours after injection of bacteria to the proximal tubule, the site’s blood flow ceases. We then measured O$_2$ levels in vivo at the infection site, using microelectrodes. O$_2$ levels dropped to 0 mmHg within 4 h post infection. Nephron cell shedding and the tissue’s actin distribution were strikingly similar to tissue damage seen in typical ischaemia experiments where the whole kidney is cut off from blood flow. We found that the O$_2$ tension was normal in tissue ∼1-2 nephrons away from the site of infection. Theoretically, O$_2$ molecules should be able to diffuse this distance, so we reasoned that the O$_2$ may be consumed on site. O$_2$ tension inversely correlated with both bacterial growth and recruited immune cells, so we isolated proximal tubule cells and measured their O$_2$ consumption. Bacteria alone were not shown to consume O$_2$ in these experiments, while proximal tubular epithelial did when bacteria were added.

We proposed that the kidney rapidly elicits local ischaemia as part of a host response to bacterial infection. Low O$_2$ concentration will have a profound effect on the immune response. For example, phagocytosis by macrophages is enhanced in hypoxic conditions [133], NF-κB can be activated through prolyl hydroxylases when O$_2$ levels are low [134], and hypoxia and innate immunity are intricately linked via their key transcription factors [135]. For example, in infections of the skin, O$_2$ pressure will be naturally low deeper inside the connective tissue, where immune cells infiltrate to reach the bacteria. Since the kidney does not have a thick connective tissue and immune cells can’t move deep into this layer, the host may need to create an artificial local hypoxic situation to enhance immune responses.

The causative agent of O$_2$ depletion may partly be the epithelium. If so, an interesting question would be to investigate how the cells deplete the O$_2$. Production of superoxide or reactive nitrogen species may explain this phenomenon, since these processes consume O$_2$, and both are implied in host responses to bacteria [136, 137, 138, 139]. Immune cells, especially neutrophils, are also a theoretical candidate for O$_2$ depletion, since they are able to produce large amounts of superoxide when activated, a process termed oxidative burst (reviewed in [140]). However, O$_2$ levels drop before their presence on the site of infection can be verified in our model. When the cells arrive they will likely increase the effect, possibly making hypoxia a combined effort.
Coagulation is a host response that protects against sepsis

The mentioned cessation of blood flow raised a number of questions about both its cause and function, and we focused our research on the non-fluorescent aggregates found in peritubular capillaries, reasoning these may block the blood flow. As we reasoned these may represent blood clots, we used real time PCR to measure activity of coagulation genes in dissected tissue (Fig. 4). Fgb, Fgl2, Serpine1, F3, Tfpi2, and Selp were shown to be upregulated 5-8 h post infection. To investigate the function of coagulation as a host response, we treated animals with the clotting inhibitor heparin. This markedly prolonged the time of intact blood flow to the site. Surprisingly, heparin treatment during infections caused rats to suffer a sharp drop in blood pressure, leading to death. Bacterial culturing showed that large numbers of bacteria had entered the systemic circulation. These results indicate the important role of coagulation as a protective mechanism in kidney infection.

A large body of literature exists on the link between inflammation and coagulation, though these papers deal mainly with coagulation as a result of sepsis [141]. Coagulation as a result of epithelial infection or inflammation thereof is less well established, but has been shown in the lung, as a result from LPS inhalation [142]. This in accordance with our data: the lung is a compartmentalized organ like the kidney, and is highly vascularized to facilitate gas exchange. Although this is speculation, organs comprised of many individual units in close proximity to the capillaries may have a benefit in closing blood supply to small infected units, allowing the organ as a whole to continue functioning. As mentioned before, both organs also have little space in the extracellular matrix, when compared to e.g. skin. Recruited immune cells thus do not have a large volume to move into; clotting may be necessary to hold recruited cells together in the blood vessel. Although leukocytes are seen in the proximal tubule among the bacteria, they do not seem to migrate immediately after arrival, as many more of them can often be seen outside of the tubule in our experiments. This may be time-frame dependent, since neutrophils have been shown in urine in longer-term UTI models [143, 144]. How coagulation stops sepsis is yet unknown, but clots may theoretically have importance as physical blockades to bacteria and as blood flow stoppers. A third role for platelets may be as sites of enhanced immune cell activation as they can induce responses in neutrophils upon bacterial recognition [145, 146].

The gene expression analysis showed upregulation of a number of genes that were annotated as involved in coagulation, and of hypoxia-inducible gene Angptl4. Surprisingly, we did not see an increase in the expression of hypoxia signature gene Vegfa. However, there may not be a need for VEGF-A protein in the inflammation, since this protein stimulates angiogenesis. Its putative increase may be repressed by the immune response. The coagulation genes we saw increased expression of were an indication of the involvement of the coagulation system, and we found an mRNA increase of the possible initiator of coagulation, tissue factor (TF), and of its pathway inhibitor gene Tfpi2. The mechanism of coagulation may thus go through the TF pathway. TF is
an initiating factor of coagulation and is not normally expressed in blood, but
becomes exposed on the surface of mononuclear cells and endothelium when
stimulated with bacterial products or proinflammatory cytokines [147, 148].
The clotting cascade may thus be triggered either from the endothelium, or
from activated immune cells that are recruited.

It is not clear how interwoven coagulation, blood flow cessation, and low O₂
pressure are in this system, but the three phenomena seem tightly intercon-
nected. The heparin experiments suggest a causal relation between coagula-
tion and a blood flow halt. Then again, we observed O₂ drops before blood
flow cessation. Even if these three processes probably depend on each other,
the intuitive rationale that coagulation leads to decreased blood flow which
leads to hypoxia may not be true. O₂ may be consumed at the site and blood
flow may be stopped by other factors than coagulation, such as local vaso-
constriction. This seems a counterintuitive possibility, as the inflammation
dogma dictates that inflammatory substances such as prostaglandins are va-
sodilators (for example, see Neisius et al. [149]). One could speculate that the
kidney may not need this increase in blood flow due to its high vasculariza-
tion, and that increasing blood flow to leaky inflamed kidney tissue may lead
to undesirable filtration to the urine of proteins. Yet if blood flow ceases at
an infected nephron, how would leukocytes enter the site of inflammation? It
should be noted that the blood flow halt is extremely local, often confined to
a part of a nephron only. Leukocytes may still be recruited at an uncongested
capillary close by, from where they possibly crawl into the site.

**Gene expression studies of early host responses**

We have shown that it is possible to analyze molecular details of host responses
by RNA analysis in this infection model in both Paper I and II. Within 8h
of infection, RNA expression analysis could show up-regulation of a number
of canonical inflammation and coagulation genes. Aside from showing proof
of principle that it was possible to measure gene expression changes in our
infection model, we could draw conclusions about the progression of the host
response.

To understand more broadly how the host tissue response was orchestrated,
we used a non-biased transcriptomic approach in Paper III. Using microar-
rays on tissue biopsies, we found ~60 significant differentially expressed genes
8 h post infection. When these data were subjected to a Gene Ontology
analysis, a number involved gene functions linked to inflammation and other
tissue processes emerged (Fig. 5). To identify which host response genes were
active in different infection models, we compared our data with that of on-
line accessible data on comparable inflammation models, limited to sets that
fit our experimental model (sterile organ, Gram-negative stimulus, <12 h).
This resulting in only three data sets may be taken as an indication of the
scarcity of early time point infection experiments done in live animals. Eight
genes were found to be upregulated in all four data sets. Since microarray
data contains a large amount of semi-significant information, we lowered the
Bayes significance thresholds for the four data sets, and compared them again, now finding 80 genes that are up-regulated in early, local host responses to gram-negative pathogens or their constituents, which we termed the “core gene expression”. This representing a 10-fold increase, false positives were avoided by the demand for a gene to be differentially expressed in all 4 sets. When we repeated the analysis replacing our 8 h post infection data set with our previously deemed non-significant 5 h post infection set, we could show differential regulation here too. We found genes that were upregulated in 5 and 8 h post infection sets, as well as genes upregulated 5 h but not 8 h post infection, showing different gene expression kinetics in tissue responses.

Comparative transcriptomic analysis could thus uncover gene regulation of lower statistical significance. As we used a 5 mm biopsy punch to separate infected tissue from the rest of the kidney, many more nephrons than the one injected are included in the analysis, resulting in background noise. To show gene expression changes before eight hours, techniques that isolate the infected site may be used to exclude background signals. If one uses e.g. laser capture microdissection [150], different cell types can even be separated based on morphology. However, this will result in a lower RNA yield, to which the downstream microarray hybridization protocol would need to be adjusted. A possibility here would be to use mass sequencing [151], in which smaller amounts of total RNA can be analyzed via sequencing.

At 8 h post infection, epithelial and endothelial cells affected by the bacteria are as mentioned relatively rare in the tissue. In the unaffected tissue surrounding the site of infection, both of these tissue cell types are overrepresented, which makes it almost impossible to detect subtle expression changes.

Figure 5: A Gene Ontology-based graph of functional gene classes found the microarray data of paper IV. Yellow to red denote increasing significance of overrepresentation of a category in the data, white - no significant overrepresentation. Adapted from paper IV.
in the affected cells. Then there is a relatively large population of recruited cells that are found in neither control nor surrounding unaffected tissue. These cells may be present in larger amounts than infected epithelium, since they do not just dock in a single layer to the endothelium, but may also extravasate and dock to each other to some extent, see for example Guyer et al. [152]. Blood vessels may theoretically be filled with multiple layers of cells whose mRNA expression profile is inherently different from the tissue cells.

Recruited cells are unable to contribute to measurable down-regulated expression of genes in the tissue if they do not carry natural antisense transcripts [153]. This brings us to the next point in our transcriptomic measurements: we find far more up- than down-regulated genes. This makes sense in that a 1000-fold local up-regulation in one infected nephron out of 1000 nephrons would result in a twofold measured up-regulation. In the case of a 1000-fold down-regulated gene, the background signal of the other 999 unaffected nephrons would effectively obscure the down-regulation. The genes we do find to have lower expression in the infected tissue may thus likely be down-regulated in the whole excised tissue, and possibly the entire kidney, due to systemic effects.

Microarray data can be typically hard to interpret due to sheer volume. Often, a number of genes are differentially expressed at high significance, followed by a large amount of genes that are marginally significant and are ignored in subsequent analyses. Amongst these, many but not all genes may be differentially expressed and are thus false negative measurements. We show that comparative transcriptomics can use semi-significant genes while avoiding false positives. As shown in Dudley et al. [154], diseases correlate across different microarray experiments, and identical diseases in different tissues correlate better than different diseases in identical tissues, which supports our results. The amount of data sets available was however small, which may have decreased analysis strength. We distilled information from data sets by comparing them in a straightforward and fast binary fashion, which disregards the information that is contained in the individual genes’ expression values and statistics. A more elaborate analysis has been done by Jenner and Young [155]. These authors showed a 511-gene core of what they termed “general alarm signal” against pathogens. In contrast to in vivo data, a large supply of in vitro datasets is available online to do these kinds of analyses. Limitations notwithstanding, our core contained ~40 genes that were not found in the “general alarm signal”, suggesting that these genes may play a role in infections in the live animal rather than in the culture dish. If more experiments on early local infection will be publicly available in the future, experimenters could derive core gene expression of multiple time points from these sets to expose gene expression interactions and relationships over time.

IFN-γ is an early responding immune modulator

The common core was an interesting finding in itself, but uninterpreted it is still not more than information. We formulated a testable hypothesis from
it: 25% of the genes found were annotated as IFN-γ-responsive. We thus hypothesized that this cytokine may be produced by the spleen, and indeed found heightened serum IFN-γ levels as well as splenic upregulation of Ifng, making it likely that this organ was responsible for IFN-γ production that led to increased serum levels. The widespread presence of IFN-γ in the body through serum will have consequences for the immune response. It has been shown that IFN-γ can potentiate NF-κB activation [156, 157], but likewise can it inhibit expression of certain inflammatory genes [158, 159]. We showed in Paper IV that IFN-γ downregulates transcription of certain neutrophil-attracting chemokines.

Figure 6: A hypothetical model of the cross-organ immune response, where local tissue reacts to infection by mounting an inflammatory response, releasing cytokines such as IL-23 in the blood. Splenocytes may react to this stimulus by secreting IFN-γ, which then can affect present and near-future inflammations.

Since IFN-γ is capable of upregulating monocyte- and T-cell attracting chemokines [160], it can be hypothesized that it steers the immune response away from massive neutrophil influx and attracts other functional cells. An interesting experiment would be to investigate which effects IFN-γ has on endothelium, immune cells, and other (immune) cells in the tissue.

For the mechanism of induction of IFN-γ production in the spleen, LPS leaking to the bloodstream would be a candidate. However, no signs of systemic inflammation were seen, and splenic expression of LPS-responsive IL-12 and IL-23 subunit genes was not upregulated. This may be due to different expression kinetics for Ifng and the interleukin genes, but it has been found in mice that the Il12a gene is upregulated in spleen 3 h post LPS injection [161]. In case whole bacteria would have leaked into the bloodstream, sepsis would have been another result, which lead to host death in Paper II. On the infection site, a variety of cytokines will be produced that can enter the circulation, and we have hypothesized that IL-23 is a cytokine that communicates renal signals to the spleen. Splenic tissue reacted in an IL-23-related fashion, since the Il17 gene, which can be induced by IL-23, was upregulated while
spleen IL23 transcripts themselves were downregulated. IL-23 can induce Ifng expression without inducing IL-17 in certain cells [162], but IL-17 itself is also capable of this induction [163].

However, we cannot exclude that more cytokines are implicated. The usual cellular suspects in IFN-γ production are T lymphocytes, and there may be a pool of different splenic T cell subsets (e.g., NKT and γδ-T cells) that react in this manner, but other cell types may too produce IFN-γ. For example, neutrophils are implied in its production in a model of renal ischemia-reperfusion injury [164, 163], and macrophages produce the cytokine in response to IL-12 and IFN-γ itself [165, 166]. Certain DC subsets are also able to produce IFN-γ in response to IL-12 [167], which we did not find up-regulated in our study.

**IFN-γ downregulates epithelial chemokine responses**

IFN-γ has been shown to both promote and inhibit transcription of different genes [168], and we have investigated its role in chemokine production by human epithelial cells in bacterial infection in Paper IV. Production of chemokine IL-8 was shown to be inhibited in both kidney and bladder cell lines. We showed that IFN-γ downregulates the production of IL-8 in an LPS-independent and transcription-dependent manner. This phenomenon has been reported in different cell types, but the mechanism has not been elucidated. We show further that transcription of neutrophil-attracting chemokines CXCL1 and IL8 is downregulated, while CXCL2 and IL6 transcription is slightly upregulated. This distinction indicates that IFN-γ does not act as a strict inhibitor of inflammation, but sooner as a modulator which steers the inflammation process in a certain direction.

We go on to show that IFN-γ exerts its effect directly and does not need *de novo* protein synthesis. The inhibiting effect is not critically dependent on key factors in the inflammatory response against bacteria NF-κB, p38 MAP kinase or c-Jun N-terminal kinase (JNK). However, p38 seemed to be the most important factor in this system, strongly downregulating the inflammatory response. Interestingly, canonical IFN-γ transcription factor STAT-1 seemed not to be the effector that downregulated IL8 expression, which may mean that a different target is activated at the IFN-γ receptor.

IFN-γ has been proposed to downregulate genes in multiple ways. Ho *et al.* proposed both the induction of an inhibiting transcription factor ATF-3, and downregulation of AP-1-mediated transcription [169]. While we can rule out actions via newly induced transcription factors, AP-1 inhibition might be the case. AP-1 dimers are composed of different proteins which can be phosphorylated by e.g. MAP kinases. Our inhibiting of JNK and p38 did not abolish IFN-γ’s effect, implying that AP-1 may not be downregulated on that level. This does not rule out that different AP-1 complexes and pathways to these complexes may be used when we inhibit kinases. Non-DNA binding transcriptional co-factors may also be the target of IFN-γ-mediated downregulation A
study of these showed inhibiting actions of IFN-γ via the sequestering of co-activators CBP/p300 [170]. These proteins were hypothesized to be recruited from protease promoters to IFN-γ-regulated promoters. Downregulation may even be a multi-pathway process and proves an interesting problem for future research.
Conclusions

A strong immune response towards pathogenic bacteria is crucial to hosts, and there is still a lot to discover on how host processes are mounted and controlled. Since most host-bacterial interactions have been studied \textit{in vitro}, a significant part of living-host responses have remained undiscovered. We have developed live imaging methods to venture into this unknown area. As we show here, live hosts respond quickly in a whole-tissue manner to eradicate bacteria. Fast shutdown of blood flow, O$_2$ restriction and coagulation are involved in defending the kidney. Transcriptomic and bioinformatic methods were used to discover unknown effectors in early host responses and resulted in the identification of IFN-\textgamma as one of these important effectors. This cytokine was found to downregulate transcription of a number of chemokines in renal epithelial cells, thus modulating the proinflammatory response. Collectively, the data show the importance of live infection models and the possibilities offered by large-scale data sets. Both will likely become more important in the future to discover yet unknown processes, possibly leading to medicine development.
Perspectives

The objective of this thesis was to answer questions about host responses to infections. Like in any scientific publication though, the research put forward raises numerous new questions. I will here try to address those that I find particularly interesting.

What the mechanism is through which oxygen levels are decreased is an intriguing question. We observed blood flow cessation and proved a possible \( \text{O}_2 \) consumption in the culture dish, but a detailed answer may be multifactorial, and not easy to investigate \textit{in vivo}. Active consumption by epithelium may be assumed to play its largest role in the first hours of the infection, when blood flow effects are not seen yet. At 3-8 h post infection, \( \text{O}_2 \) may be consumed by recruited cells, too. It could be investigated whether epithelium reacts to bacteria with a strong respiratory burst. Epithelial cells have been reported to respond to bacterial products with superoxide production, although in these experiments, superoxide levels were measured after 24 h \[171\]. \textit{In vivo}, NADPH oxidase-blocking agents may be used to prevent such a burst. This sort of experiments is not without problems though, as delivery of blocking agents will conventionally be throughout the body and may tamper with signaling processes. Conditional knock-out mice may be helpful here: one could screen mice with knock-outs of the different \( \text{Nox} \) and \( \text{Duox} \) genes that code for NADPH oxidases. The function of low \( \text{O}_2 \) levels for the host response may also be researched. If a fast burst can be shown to be induced \textit{in vitro}, biochemical dissection to find signaling targets of this pathway can be pursued.

The triggering of the coagulation response as an innate immunity mechanism is another finding that may be investigated further. As stated, coagulation cascades can be started by proinflammatory agents. Conditional knock-outs or bone-marrow transplants may be used to dissect the specific cellular origins and mechanisms of this phenomenon. How coagulation works to restrain bacteria from entering the blood is also a question that results from our work. This is somewhat harder to approach with conventional molecular biology tools, as inhibiting specific components of the coagulation pathway may affect the inflammation response itself. For example, it can be hypothesized that coagulation leads to a stickier complex of inflamed cells, which would increase the recruitment of immune cells. If coagulation affects inflammation and vice versa, one cannot easily block either part to find out what stops the bacteria. Here, a more subtle method is desired. Host component concentrations may be increased or decreased in small steps, one could even perturb multiple components at the same time (e.g. increasing platelets and decreasing neutrophils). This would probe the inflammatory network more gently than knock-outs.

It is currently unknown in what ratios immune cells are recruited to a site
of infection. Immunology comes with powerful tools to define different populations of immune cells by their surface receptors and behaviour, and new subsets are still being discovered. However, one does not simply FACS a local infection. The infections we induce are so small that it may be hard to isolate enough recruited cells to analyze. It may also be worth asking the question whether subset definitions are of any interest, when one realizes that man is not a rodent and is equipped with different amounts of immune cells in the bloodstream in the first place. Still, the immune systems' similarity justifies comparisons on a less detailed scale than the exact amount of certain cell types. For example, it would be interesting to know if one could manipulate the concentrations and amounts of certain factors in the system to test robustness and dependencies of the host response. Manipulating concentrations forms a somewhat subtler tool than knock-out studies, but is as such also harder to attain. Perturbing the amount of blood-borne immune cells can be done, but to de- or increase protein expression \textit{in vivo} is difficult. A solid \textit{in vitro} system may be developed, which then can be implemented in the live animal by means of e.g. bone marrow transplants or knock-ins. Here too, having multiple readouts may be of importance.

We found a systemic presence of IFN-\(\gamma\) in response to infection. An immediate question to ask is what cells generate this expression, and one can use a technique like FACS to determine this. However, this should probably be done in mice, since the availability of antibodies and knock-outs for mice is far greater than for rats. When making this transition, it can also be researched if this is a response shown for more systems than our induced kidney inflammation, which should not be a technical problem if an early local infection model is used. Combining serum cytokine analysis with knock-out animals would help to identify the inducing agent(s). What may be of equal interest is how IFN-\(\gamma\) contributes to both the ongoing immune response at the infected nephron, and a putative near-future immune response. Note here that kidney infections usually stem from a bladder reservoir, from which multiple nephrons can be infected at different time points. Since all bloodborne cells and endothelium will be exposed to the IFN-\(\gamma\), they may respond differently than when encountering their first infection. Indeed, our work in Paper IV points out that IFN-\(\gamma\) can downregulate neutrophil-attracting chemokine production. The effects of this on infection may be researched by imaging the infection using knock-out animals, in which the readout may be e.g. neutrophil behaviour.

The mechanism behind IFN-\(\gamma\)'s effect on \textit{IL8} transcription would also require further research. As this is a problem of more molecular-biological nature, it would require a similar approach. Brute-force perturbation methods such as siRNA screens may work, but are costly and may miss their target if redundancy exists. A more viable approach may be to use microarrays or mass-sequencing to identify IFN-\(\gamma\)-downregulated genes, after which these can be clustered according to promoter/enhancer sequences. From there on one could work bottom-up. Mass-sequencing would also possibly identify
microRNAs that could negatively affect transcript levels. Another systematic approach would be to determine IFN-γ’s effects on histone modifications in the IL8 promoter/enhancer region, using chromatin immune precipitation (ChIP), which would yield information on what signal-transduction level is affected by IFN-γ.

I would like to plead here for a truly integrated approach to the questions described above. Whatever the method used for solving them, questions concerning complex networks require combining a larger number of measurements and a multivariate analysis. This implies using different input stimuli in various concentrations and combinations, and perturbing components between input and output. The output readout should be captured in multiple ways, too. For example, when researching coagulation and sepsis, measure not only numbers of translocated bacteria, but also size, permeability and contents of clots. The approach may not be the easiest, and the amount of data generated will be vast, but a reliable network model can be built from these numbers.

In conclusion, I have in this thesis hardly lifted more than a corner of the veil that covers bacterial inflammation. Many problems, both large and small, remain to be researched. I have only described a few questions that are derived from this work here, and know that the solutions to these will in themselves provoke new questions. The amounts of work to be done show that the field will remain an exciting one for at least the coming years.
My thesis for the layman

Bacteria are potentially dangerous for the human beings that they infect (hosts), as they can damage host organs like the kidney. To combat infections, hosts are equipped with defense systems to detect, isolate, and kill invading bacteria. When cells in e.g. the kidney detect bacteria, they immediately alert their neighbours. They also call in the cavalry, which consists of white blood cells. The blood vessels close to bacteria change from blood-conducting tubes into velcro-like sites where white blood cells stick to as they pass. These recruited cells will then crawl into the space where bacteria are, and try to kill the bacteria. The reaction of the kidney’s cells (or those of another organ), blood vessels and white blood cells is termed inflammation, which becomes visible to us for example when bacteria infect our throat. The tissue swells, turns red, and hurts because of the changes in blood vessels and recruitment of white blood cells.

The battle against infection can be longer or shorter, but its outcome and how it is fought depend on what happens in its first hours. It is not easy to study early events, since at that point there will be fewer bacteria and host responses than at later time points. Especially in the kidney, which can be imagined as an enormous amount of connected tubes, it is not easy to know in what tube the bacteria are. That is why we have designed a kidney infection model system to study these early time points. We know exactly where and when we inject bacteria in a kidney, so we know where and when to look. To look, we use a special microscope that allows us to look inside the kidney from the outside, without cutting it up. Using this so called multiphoton microscope, we can look at the bacteria while they grow inside the kidney, and while immunity combats them.

We found that the host responds to bacterial infection really fast. The kidney brings down oxygen levels at just that very small site where bacteria are. This may be a signal for immune cells to work harder. We also found that the blood in vessels that surrounded the infection formed clots, just as it does when you cut yourself. When we made blood clotting impossible, the bacteria found their way out into the blood stream and killed the animal. The clotting of blood may thus be a mechanism to isolate the bacteria from the rest of the body. Clots may also be actively involved in recruiting immune cells. When clots form on a blood vessel, the vessel’s velcro-like surface gets a different structure, which may be beneficial for immune cell recruitment.

All cells that are involved in this inflammation, from kidney cell to white blood cell must basically quit whatever they were doing before the inflammation and start combating bacteria. Just as we would change from eating dinner to brushing teeth by exchanging cutlery for a toothbrush, the cells must change the proteins that they use. The difference is that we have our tools ready in a cupboard, while cells have to make their proteins from scratch. A kidney
cell for instance, must stop making a certain urine-filtering protein, and start making one that alerts other cells. Of course some proteins are so vital for the cells that they cannot stop making them, just as we may keep our glasses on during both dinner and brushing teeth. When making new proteins, cells use their DNA, stored in the cell’s nucleus, as a blueprint. The DNA codes for the individual proteins. First, the DNA that codes for the necessary protein is copied, or transcribed, to mRNA, a messenger molecule that transports the DNA code out of the nucleus to a cellular protein factory termed ribosome. The mRNA code is then read and translated into a protein by the ribosome, after which the cell can use its new protein tool.

It is not easy to measure all proteins which the host makes in an inflammation, but we can easily measure mRNA quantities. We have measured every protein’s corresponding mRNA simultaneously to indirectly see which proteins are being made during a host response to kidney infections. We showed that these responses have similarities with responses in lung and brain. While in all these responses host probably make several hundreds of proteins, we discovered that 80 mRNAs were made in all of the mentioned inflamed tissues. The nature of these 80 mRNAs led us to believe that a protein called IFN-γ was important in signaling to the cells what proteins they should make. We found that it was actually made by the spleen, which was not infected. So, the kidney senses infection and signals to the blood vessel to recruit immune cells. But to alert the rest of the body, it also sends out an alarm signal that other organs like the spleen respond to. The spleen contains many immune cells that notice this signal, and releases a protein in the blood. The protein, IFN-γ then travels to the kidney and instructs the cells there what to do against the infection. Some of these instructions seem to tell the kidney to slow down the inflammation process, which may mean that IFN-γ is part of a communication system that protects the kidney from an overambitious and dangerous inflammation response.

The work presented in this thesis shows that we can discover unknown host responses when working with live models instead of isolated cells. Isolated cells in a culture flask can be cheap, fast and animal-friendly models, and yield reliable data. This is a reason why many discoveries have been made in these cells. However, when using a living host as a model, effects of other organs than the infected one are also allowed to play a role and can be measured. Much about the immune response to bacteria is yet unknown, and more high-resolution living models may be developed over the coming years to study this.
Acknowledgements

Have you read the rest of the thesis? Chances are that you haven’t ($P < 0.05$), but neither this thesis, nor myself would not have been the same without the following people. Thanks to:

Agneta Richter-Dahlfors, for accepting me as a Ph.D. student, and giving me great independence. It’s been a very educational experience. Tack framförallt för att du alltid såg till att vända min pessimism till optimism.

Mikael Rhen, my co-supervisor, for all scientific discussions we’ve had and the tremendous help in writing that paper. Du är som en vandrande pubmed, och inspirerar mig ständigt till nya experiment.

Thanks to the past and present members of the ARD group: Arley for Spanish lessons and medical expertise, Camilla for the take-no-shit attitude och tips av den skolan, Daniel and Jakob for that table at Alinea or Noma that we’ll book when we get rich. Ferdinand, we should meet up in Singapore for dinner, suit you Sir John, for the Visual Basic course. Kader, for really good akutkaffe, conversation and a persuasive attitude concerning food and drinks. Pepparkaka? Kalle för jazz, öl, alla kreativa planer vi hade och har, och tre nyärsfester. Nästa gång bjuder vi Thorsten också. Karin, för din humor och alla kakor du tar till kontoret som jag äter upp, och för att jag lärt mig mycket om att vara organis erad, Keira, for living through the same bacterial and rat frustrations from day 1, for unabashedly correcting my deteriorated English, and for your excellent taste in music of course. KJ, för din beröknings av min svenska med ’goffa’ m.fl. och din skådistalang, Klas, I haven’t benefited enough from your lab-competence, but moreso from your humour, Lisa, för att du visade runt mig i Indianapolis och inte minst för att du ordnade bostad åt mig när jag kom hit utan att du visste vem jag var. Margret för att du håller ordning på chefen (och på oss förstås), Monica för paper-editing, och för att du inte klagade på att jag stod längst bak och mumlade i karaoke-kören. Tack Peter, som var den enda som uppskattade foul cursing in the lab och som introducerade mig till den svenska konferens-traditionen. Sindhu, for serious talk, helpful tips and a sharp sense of humour, and Susanne, for nerder y und Gugelhubschrauber.

The Andreas, Björn, and Gabriella labs, including the XYZ-team, Marco, Cosimo, Ann-Sofie, Sergey for a good atmosphere, a coffee machine and a healthy scientific attitude.

Dr. Bruce Molitoris and the staff at the O’Brien Center for Advanced Renal Microscopic Analysis, for making my stay a memorable one and for a nice and fruitful collaboration. Special thanks to my kidney and microscopy experts/co-authors Dr. Tanner and Ruben Sandoval — but not for introducing me to Taco Bell and their ’meat’ products.
Örjan Källskog, who taught me most of what I now know about the kidney, which was just a collateral effect of our collaboration. Efter mycket snack har jag nu också äntligen klarat Vikingarännet. Also thanks to co-author Fredrik Palm, for those oxygen experiments in paper II.

All members of the IMO-Train program, Ute, Agaristi, Claudia, Katharina, Kristina, Nicolas, Sönke, Speranta, and Relu, for organizing a pretty great grad. student program with lots of seminars and discussions.

Everyone I met at MTC, but especially Alexander, for a shared biking passion, Anna för father Ted, Borat och ’Macbeth’!, Clemens for espressos and precisely determining beverage quality grades, Fedor for importing Gzhelka, sushi-Helena för quality-sushi och skitsnack, Martin för kaffe, nerdtalk, och att vara min privata Debian/R-helpdesk, and Uffe för att du introducerade mig till the world of microarray (men inte för dina snus-doftspår i arrayrummet).

Monica Thelestam’s lab for sharing a lab, offices and journal clubs. Teresa, for your great expertise, company, and always-ready attitude for my questions and for being fresca come una rosa. Lina, för att jag fick öva båda italienska och finska på din fest och för att dela labb var ett stort nöje.

Lots of people at Neuroscience, but most importantly Ola and the rolling histones (Amilcar, Anna, Erik, Ezra, Giulia, Gonçalo, Karolina, Michalina, Shirin, Therese, and Tobias), as well as Mia, Marta and Richard for sharing a lab, Elvis-fridas, ever-refilling candiesupplies, and lab-advice whenever I needed it. Special thanks to Nina and dr. K***** for helping me stumble through ChIP protocols.

Everyone I ever had a coffee or a lunch with the last six years and shared feeling like we were in an episode of Ph.D. comics. You know who you are.

JW, instrumental for finding an internship, a job, and a wife. Dat emailtje heeft zich ruim terugbetaald.

The Dutch connection, especially Bart and Marieke. Slaapplaats, entertainment en altijd tijd hebben voor mijn laat-aangekondigde bezoekjes heb ik als een groot privilege beschouwd.

Irja, för alla management-tips, god mat, trevlig sällskap, gratis hotellrum på landet and what not. Tack.

Natuurlijk papa, mama, Sietse en Arne voor de broodnodige support en spa-vakanties in het exotische Abbekerk. Ik moet toch vaker langskomen.

Min kära Carolina, för mycket mer än summan av allt.
Bibliography


