Local proliferation of Ly6C+ monocytes in inflamed tissues and during bacterial infections

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List of abbreviations

- BM Bone marrow
- BSA Bovine serum albumin
- BrdU Bromodeoxyuridine
- CFU Colony forming unit
- CLC Cardiotrophin-like cytokine
- CNTF Ciliary neurotrophic factor
- CT-1 Cardiotrophin-1
- cAMP Cyclic adenosine monophosphate
- CKD Chronic kidney disease
- CXCL2 Chemokine (C-X-C motif) ligand 2
- DAMP Damage associated molecular patterns
- DC Dendritic cell
- DTR Diphtheria toxin receptor
- FCS Fetal calf serum
- ECM Extracellular matrix
- EGFP enhanced green fluorescent protein
- FimH Fimbrial adhesion molecule H
- HSC's Hematopoietic stem cells
- IBC Intracellular bacterial colonies
- IL-1 Interleukin 1
- IL-4 Interleukin 4
- IL-6 Interleukin-6

- KSHV Kaposi's sarcoma-associated herpes virus
- LIF Leukemia inhibitory factor
- LPS Lipopolysaccharide
- LRR Leucine Rich Repeat
- MHCII Major histocompatibility complex II
- MMP-9 Matrix metallopeptidase 9
- MP Macrophage
- NFkB nuclear factor kappa-light-chain-enhancer of activated B cells
- OSM Oncostatin M
- PAMP Pattern associated molecular patterns
- PBS Phosphate Buffered Saline
- PFA Para formaldehyde
- PMNs Polymorphonuclear cells
- RT Room Temperature
- TIMP-1 Metallopeptidase inhibitor 1
- TLR Toll like Receptor
- UPEC Uropathogenic E. coli
- UTI Urinary tract infections
- UUO Unilateral ureteral obstruction
- vIL-6 Viral homolog of IL-6

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Zusammenfassung

Monozyten vermitteln antimikrobielle Immunantworten und regulieren inflammatorische Prozesse. Es wird angenommen, dass diese Zellen nicht-proliferierende myeloide Zellen sind, dessen Vorkommen im peripherem Gewebe von der Rekrutierung aus dem Blutkreislauf abhängig ist. Weiterhin differenzieren Monozyten nach dem Eintritt in die Gewebe in Makrophagen und es gibt experimentelle Hinweise, dass Makrophagen während einer Infektion proliferieren können, um so ihre Population zu vergrößern. Diese Erkenntnisse stellen das Dogma in Frage, dass Makrophagen terminal differenzierte Zellen sind. Es ist allerdings unbekannt, ob auch Monozyten nach Gewebeeintritt proliferieren können. Diese Proliferation könnte die Menge der Monozyten erhöhen, sodass eine verringerte Rekrutierung von Monozyten notwendig ist. In der vorliegenden Arbeit wird gezeigt, dass rekrutierte Ly6C+ Monozyten im Mausmodell der Blaseninfektion und in der LPS-induzierten Peritonitis proliferative Fähigkeiten besitzen und die proliferierenden Monozyten maßgeblich an der Infektionsabwehr beteiligt sind. Im Gegensatz dazu waren nicht–proliferierende Monozyten in der Lage neutrophile Granulozyten zu phagozytieren, um die Entzündungsreaktion zu verringern. Zuletzt konnte experimentell gezeigt werden, dass das pleiotrope Molekül IL-6 die Proliferation von Ly6C+ Monozyten induziert, welches die bedeutende Rolle dieses Moleküls und der Akkumulation von Monozyten in inflammatorischen Prozessen erklären könnte. Diese Daten zeigen die proliferativen Fähigkeiten von Monozyten während der Blaseninfektion und identifizieren IL-6 als das Schlüsselmolekül, welches ein wichtiger Regulator in diesem Prozess darstellt.

Summary

Blood monocytes are present throughout the body. These cells mediate host antimicrobial defenses and are implicated in almost every inflammatory process. It is accepted that monocytes are non-proliferating myeloid cells and their abundance in peripheral tissues depends on the recruitment from the circulation. However, there is emerging evidence that macrophages can be rescued from cell cycle arrest and actively proliferate during infections to increase population density. These findings may contribute to a novel concept that monocytes are not terminally differentiated cells without the capacity to locally proliferate. However, the proliferation of blood monocytes has not been studied so far and the contribution of recruitment versus local proliferation for the presence of Ly6C+ monocytes in inflamed tissues also needs to be defined.. Furthermore, the function of proliferative monocytes during infection and inflammation is unknown. IL-6 is a pleiotropic cytokine that is known to regulate inflammatory processes and play an essential role during bacterial infections. Role of IL-6 in regulation of monocyte accumulation during bacterial infections is also unknown. This thesis demonstrates the proliferative capacity of recruited Ly6C+ monocytes in a murine model of urinary tract infection and in LPS-induced peritonitis. IL-6 trans-signaling was identified as the key pathway that regulates the proliferation of Ly6C+ monocytes in both inflammatory models. Non-proliferating monocytes phagocytized matured neutrophils, whereas proliferating monocytes critically contribute to the defense against infection. These data reveal the process of monocyte proliferation during bacteria infected tissues and identify IL-6 as the key molecule that regulates this proliferation.

1. Introduction

1.1 Urinary Tract Infections

Urinary tract infections (UTI) are one of the most common bacterial infections worldwide. They are commonly caused by a uropathogenic strain of E. *coli* (UPEC). UTIs result in cystitis (infection of the bladder) and once the bacteria ascend to the kidneys, it leads to pyelonephritis (infection of the kidneys) and bacteriuria (infection of the urine). Recurring cystitis and pyelonephritis result in scarring, fibrosis (of the kidney and bladder) and renal failure and in a number of cases even death. Women are significantly more affected by UTI than men and it has been reported that at least half of the women experience at least one episode of UTI in their lifetime (Foxman, 2002). UTIs result in more than 1 million hospital visits every year in the US and more than 150 million cases worldwide (Hooton and Stamm, 1997; Moriel et al., 2016; Russo and Johnson, 2003). Some patients are known to be more susceptible than the others and especially children, sexually active women, pregnant women, elderly people and patients with catheters, diabetes, multiple sclerosis or HIV are more susceptible to UTI (Foxman, 2002). Complications arise from recurring UTI, which can observed in over 20% of women who experienced UTI (Hooton and Stam, 1991). Also, complications also arise from antibiotic resistance of the bacteria. The current regime of treatment of UTIs is with antibiotics that can result in alterations in normal microbiota and increasing multi drug resistance. High infection frequencies, prevalence, recurrence and increasing antibiotic resistance result in very high economic burden. Due to these implications extensive research is required in the field.

Pathogenesis of UTI in the bladder

The mode of action of UPEC has been extensively studied. Adherence of the bacteria to the bladder epithelium is a key event in the pathogenesis of UTI. Adherence is mediated by pili and results in colonization and invasion of the UPEC in the bladder (Flores-Mireles et al., 2015; Mulvey et al., 2000a). Once the uropathogens have invaded the uroepithelium, they release toxins and proteases to release nutrients from the host cells. Out of different types of pili, type 1 pili (Figure 1) have been shown to be involved in adhesion and

invasion of UPEC (Martinez et al., 2000; Mulvey et al., 2000b). Type 1 pili are tipped with adhesin called Fimbrial adhesin H (FimH) (Jones et al., 1995), which recognizes mannosylated uroplakins and integrins with stereo chemical specificity (Eto et al., 2007). Binding of FimH capped type 1pili results in activation of Rho GTPases that causes actin rearrangement and internalization of UPEC by a zippering mechanism (Martinez et al., 2000). Invasion and colonization of UPEC result in formation of intracellular bacterial colonies (IBCs), which resembles a biofilm. Formation of this biofilm has been shown to define the pathogenesis of infection and its recurrence (Mulvey et al., 1998). Growth of the bacteria in a biofilm-like manner results in a highly organized, metabolically active bacterial community that is difficult to remove by anti microbial agents. These IBCs can also switch to a quiescent phase that persists intracellularly for a long period (Justice et al., 2006). In this intracellular location, the bacteria remain protected from antibiotics and hosts´ immune cells (responses).

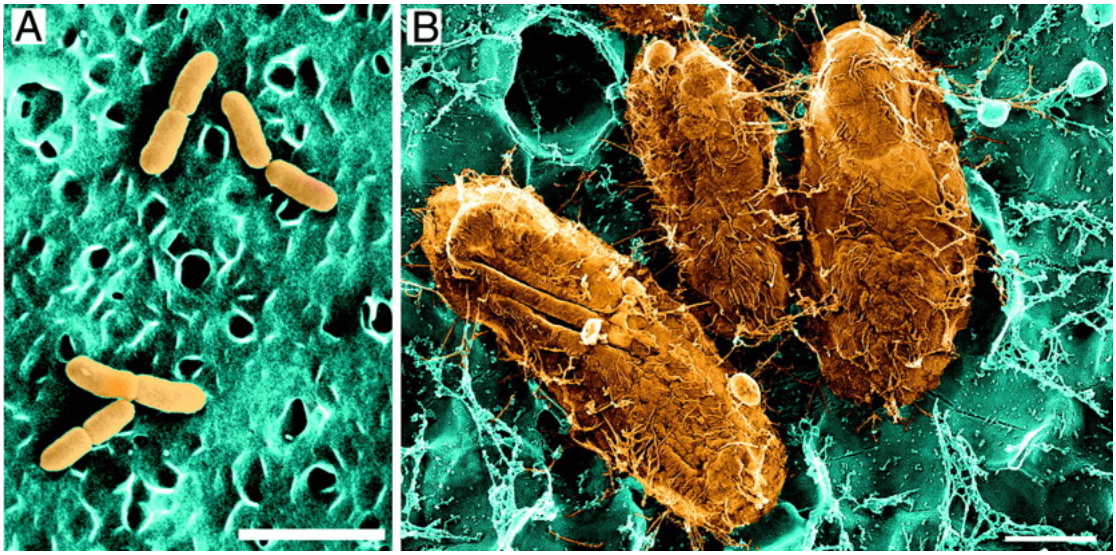

Figure 1: Structure of UPEC.
C57BL/6 were inoculated with type 1 piliated UPEC. (A) Scanning electron microscopy image of UPECs. (B) Type 1 pili mediating bacterial attachment at high resolution. Bars (A) 3μm, (B) 0.5μm. (Mulvey et al., 2000b)

Our body also has an innate defense mechanism to prevent invasion and colonization of UPECs. This is mediated by Toll-like Receptors (TLR's), which are a family of trans-membrane proteins that recognize pathogen associated molecular patterns (PAMPS). They are characterized by the presence of

Leucine-rich repeat (LRR) domain. So far, 11 TLR's have been reported in mammalian species namely TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, TLR11 and TLR13 (Kawai and Akira, 2006b; Oldenburg et al., 2012). Activation of the TLR pathway results in transcription of host defense genes. The role of TLR's has also been extensively studied in UPEC infections. It has been shown using TLR4-/- mice, that absence of TLR4 (which recognizes LPS), results in increased susceptibility to this infection and inability to clear it (Schilling et al., 2002). Moreover, it was also shown that TLR11 plays an important role in defense against UPECS. TLR11 is highly expressed in both bladder and kidneys, where it is responsible for initiating an appropriate innate immune response (Zhang et al., 2004). Activation of the TLR pathway has been well studied. The extracellular domain interacts with ligands. The intracellular domain triggers signalling pathways to activate transcription of target genes. For example: Upon recognition of bacteria, TLR4 along with its co receptor CD14 initiates MyD88-mediated signaling, resulting in activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) (Fischer et al., 2006; Kawai and Akira, 2006a; Miyazaki et al., 2006). Translocation of NF-kB into the nucleus then results in transcription of cytokines such as IL-6 and IL-8. These are the majorly expressed cytokines in the urinary tract post infection (Agace et al., 1993). TLR4 has also been shown to cause bacterial expulsion where cyclic adenosine monophosphate (cAMP) is produced and acts as a powerful trigger for exocytosis of intracellular UPECs (Bishop et al., 2007). TLR4 thus plays several functions in defense against UPECs. Despite these innate defense mechanisms, UPECs are still able to evade and invade in the uroepithelium.

1.2 Pathogenesis and innate immune response in Pyelonephritis

Pyelonephritis (PN) is the infection of kidneys and is caused when bacteria from the lower urinary tract ascend into the kidneys. PN is one of the most common forms of kidney infections caused by ascension of UPEC from the bladder. Acute PN results in symptoms such as nausea, fever, renal scarring and fibrosis. Renal fibrosis can ultimately lead to renal failure and death (Imig and Ryan, 2013).

Neutrophils are the first innate immune cells to be recruited to the kidneys post infection. This recruitment is mediated via production of chemokine called Chemokine (C-X-C motif) ligand 2 (CXCL2) from the kidney tubular epithelial cells (Godaly et al., 1997). Matrix metalloproteinase 9 (MMP-9) and Metallopeptidase inhibitor 1 (TIMP-1) have also shown to be up regulated in the urine of patients suffering from PN, indicating a role of these enzymes in the pathology of this disease (Chromek et al., 2003). Since dendritic cells (DC) are one of a major cell type in the kidneys, their role has also been investigated during PN (Rogers et al., 2014). Phenotypically, renal DCs are CD11c+ and can be further characterized into CD103+ or CD103-. They are known to be the key factors involved in initiation and propagation of a renal disease. DCs are the most effective inducers of adaptive immunity, through activation of T cells (Steinman and Cohn, 1973). DCs have also been shown to induce adaptive immunity against UPEC associated PN by detecting the UPECs and recruiting neutrophils to the site of infection so that they can perform phagocytosis of the pathogen (Tittel et al., 2011). Conditional depletion of DCs in CD11c-DTR mice by application of diphtheria toxin receptor (DTR) has also been employed to study the role of DCs in UTI. Depletion of DCs resulted in attenuation of kidney damage in a model of nephrotoxic nephritis (NTN) (Hochheiser et al., 2011a). Role of renal macrophages has also been investigated. Renal macrophages have been shown to be pathogenic by depletion of renal resident macrophages using clodronate liposomes in several renal injury models (Lee et al., 2011). On the other hand protective role of renal macrophages has also been described where they produce anti-inflammatory molecules like IL-10 and extra cellular matrix components (ECM) in different renal disease models (Guiteras et al., 2016).

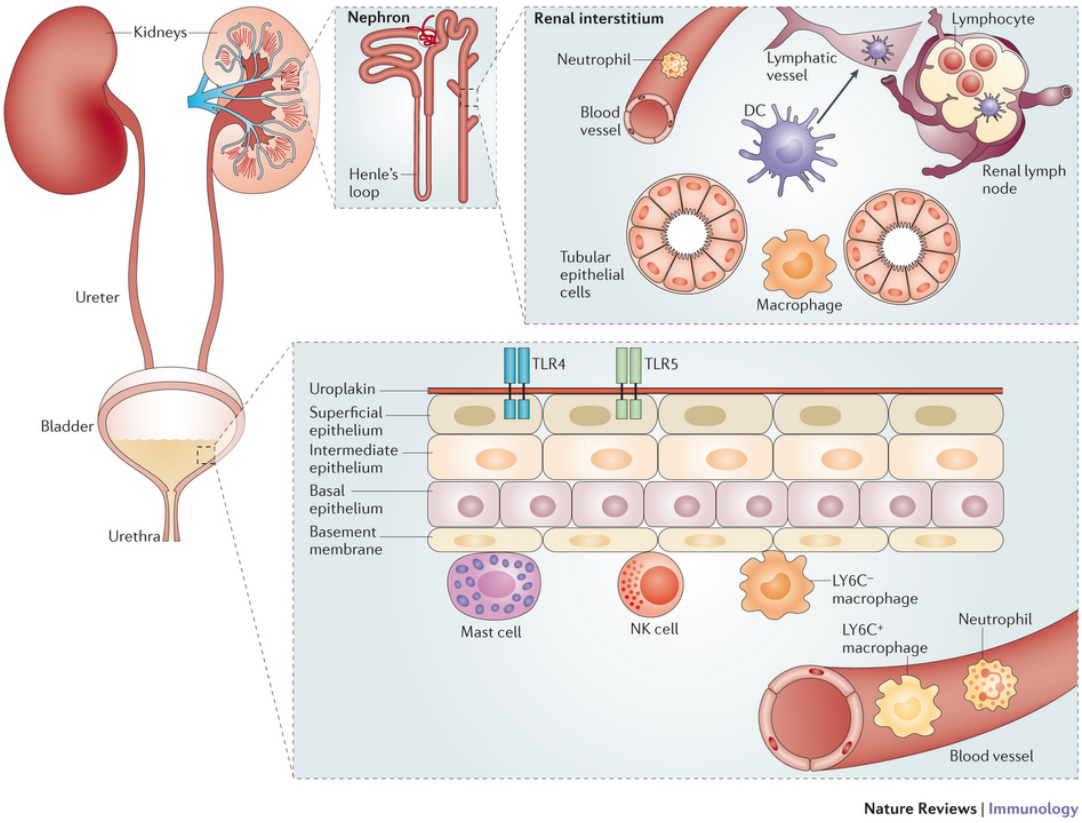

Figure 2: Structure and Organization of immune cells in the urinary tract.
The urinary tract comprises of a bladder that is connected to the kidneys with a ureter. The bladder itself is lined with several stratified layers of epithelium that acts as a major line of defense. The kidney on the other hand is divided into cortex and medulla. Several thousands of nephrons that are the urine producing functional structures span through out the cortex and medulla. The major resident immune cells in the bladder are mast cells and Ly6C- macrophages. These macrophages help in the recruitment of Ly6C+ monocytes and neutrophils and in sensing of the infection/pathogen. The major resident immune cells in the kidneys also include dendritic cells (DC) along with resident Ly6C- macrophages (Abraham and Miao et al, 2015).

However, the pathology of PN is still not completely understood. The role of phagocytes like neutrophils and macrophages in these infections is still unclear. A recent study revealed an important and coordinated function of macrophages and neutrophils; the two major phagocytes during UPEC infection of the bladder. They show that Ly6C- macrophages act as sentinels/coordinators, Ly6C+ macrophages act as helpers/advisors and neutrophils act as final anti bacterial effectors cells (Schiwon et al., 2014). Whether such findings extend to PN needs to be investigated.

1.3 Phagocytes

White blood cells or leukocytes are a diverse group of cells that circulate in the blood and are recruited to the sites of infection. Leukocytes are thus the mediators of body's immune response. One subset of leukocytes is the mononuclear phagocyte system that consists of phagocytic cells such as monocyte and macrophages. Phagocytes are cells that take up (phagocytose) dead cells, bacteria or any other debris to protect our body. Their name comes from the Greek word *phagein*, "to eat" or "devour", and "-cyte", the suffix in biology denoting "cell". Other professional phagocytes include neutrophils and dendritic cells.

Neutrophils are the major professional phagocytes in our body. They are short-lived and highly motile. Neutrophils may be sub-divided into segmented neutrophils and banded neutrophils. They form part of the polymorphonuclear cells family (PMNs) together with basophils and eosinophil and play a major role in innate immunity. They are one of the first responders that are recruited to the sites of inflammation.

Other groups of phagocytes are the monocytes and macrophages. Monocytes and macrophages are a heterogeneous population of phagocytic immune cells, which are present in almost all tissues and perform diverse functions from immunity, metabolism and tissue repair. While monocytes are known to play an important role during inflammation, macrophages are involved in development and maintenance of tissue homeostasis. Macrophages acquire distinct tissue-specific characteristics and functions, which can be seen in the liver (Kupffer cells), brain (microglia cells), spleen (red pulp macrophages) and in the lung (alveolar macrophages).

Monocytes

Blood derived monocytes can be classified as inflammatory Ly6C+, CCR2+, CX3CR1^{int} and patrolling Ly6C–, CCR2–, CX3CR1^{hi} monocytes (Auffray et al., 2007). Both types of monocytes have been considered important, because

they leave the blood to infiltrate infected tissues (Auffray et al., 2007; Schiwon et al., 2014) (Figure 3). This recruitment has been shown to be CCR2-dependent (Serbina and Pamer, 2006).. Upon recruitment, these monocytes mediate their effector functions and differentiate into macrophages and dendritic cells (Geissmann et al., 2010; Gordon and Taylor, 2005). The importance of these recruited monocytes has been demonstrated using depletion methods such as clodronate liposomes, where the absence of these cells results in reduced chemokine, cytokines secretion and altered recruitment of neutrophils (Ajuebor et al., 1999; Cailhier et al., 2006; Duffield et al., 2005).

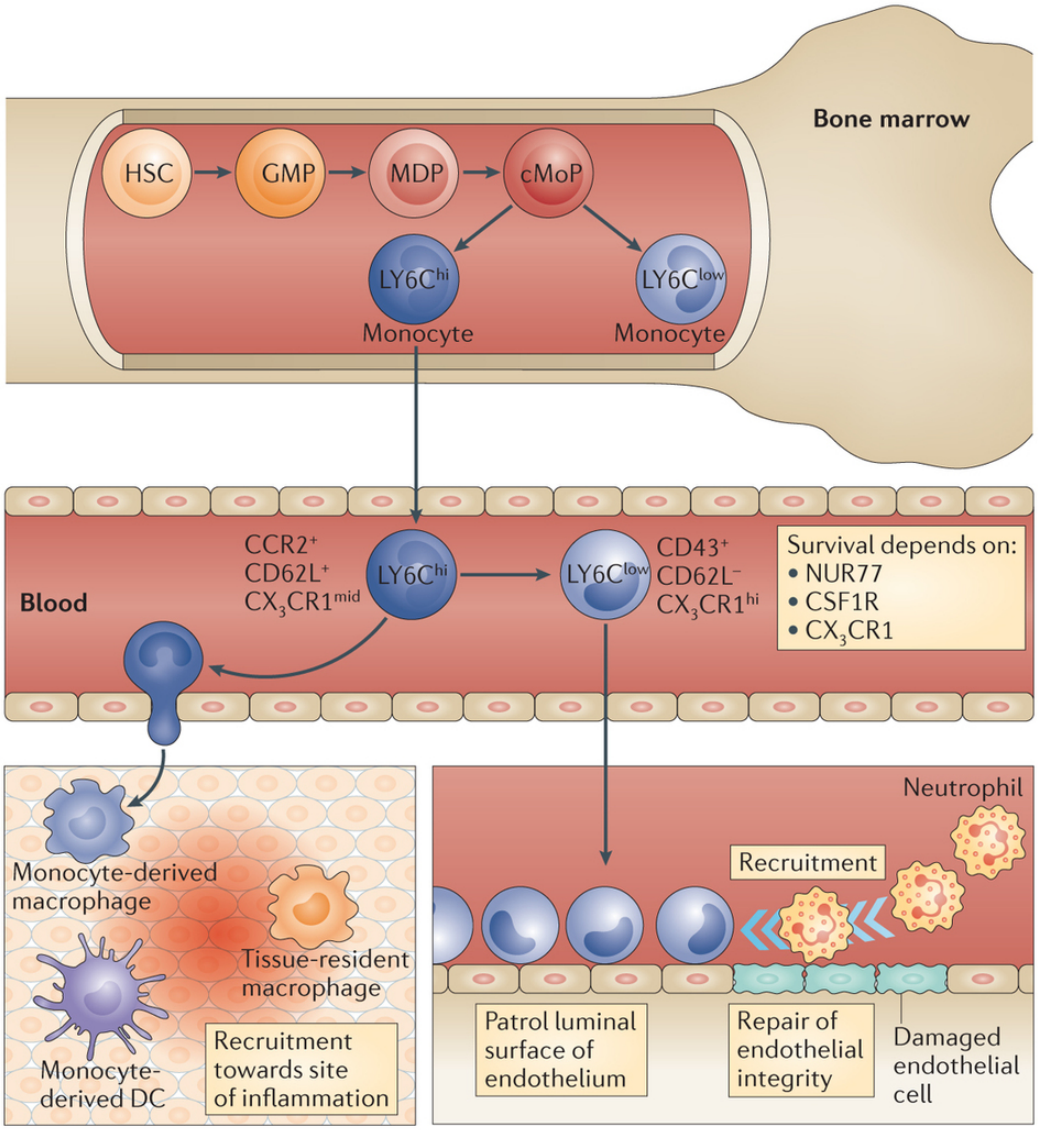

Figure 3: Origin of monocytes.

Monocytes are constantly generated in bone marrow from hematopoietic stem cells (HSCs). These cells develop into two major cell types, namely Ly6C+ and Ly6C- monocytes, which emigrate into the blood and into inflamed tissues (Ginhoux and Jung, 2014).

Macrophages

Ilya Metchnikoff first discovered macrophages in late 19th century as 'the big eaters'. It has been a central dogma in immunology over 40 years that hematopoietic stem cells (HSC's) give rise to tissue resident macrophages (van Furth and Cohn, 1968). This dogma has been revised by the recent finding that tissue macrophages can also originate from embryonic progenitors (Perdiguero et al., 2014; Schulz et al., 2012; Yona et al., 2013). This was demonstrated using Myb-deficient mice that lack development of hematopoietic stem cells. While HSC development completely depends on the transcription factor *Myb*, yolk sac progenitors develop independently of *Myb* (Schulz et al., 2012). Yolk sac macrophages appear in the blood islands at E8.5/E9.0 of the embryo development and disseminate following the establishment of blood circulation through the tissues. At around E10.5, definitive hematopoiesis is initiated giving rise to all major hematopoietic lineages (McGrath et al., 2015a; McGrath et al., 2015b; McGrath and Palis, 2005). This new understanding on macrophage origin also raises the question whether macrophages from different origins have different functions, which further emphasizes the complexity and diversity of tissue macrophages. Each organ has its unique composition of hematopoietic-derived or embryonic-derived macrophages. Comprehensive transcriptional analysis of resident macrophages by the ImmGen consortium and using fate-mapping techniques has demonstrated that macrophages obtain diverse functions based on tissue niches (Epelman et al., 2014). . However, there is still a need to develop tools for understanding the functions of infiltrating monocytes versus resident macrophages.

Macrophage proliferation

Our body is continuously faced with challenges and physiological ageing that warrants the need for constant replacement of cells. This is also true for cells

of the immune system. Mature differentiated cells are considered to have lost their proliferative capacity and hence there is a constant need for their replacement. This replacement is done by stem cell progenitors, which have self-renewal capacity.

It was previously believed that tissue macrophages are terminally differentiated cells, but recent studies show their self-renewing capacity. There is evidence that both, Langerhans cells and brain microglia cells, can self-renew by local proliferation in adult mice (Epelman et al., 2014). Using Ki67 and BrdU (Bromodeoxyuridine), which are specific markers for proliferation, it has been demonstrated that tissue macrophages can maintain their numbers by local proliferation with little contribution from circulating Ly6C+ monocytes (Auffray et al., 2007) (Auffray et al., 2007; Schiwon et al., 2014). However, there is emerging evidence that Ly6C+ monocyte proliferation establishes a local inflammatory monocytic pool (Geissmann et al., 2003), indicating that cell cycle exit can be overcome. Although local macrophage expansion appears to be a mechanism to prevent inflammation through the recruitment of blood monocytes (Geissmann et al., 2010; Gordon and Taylor, 2005), contribution of these proliferating monocytes in anti-bacterial immune responses is controversial.

Local proliferation has recently been shown to sustain macrophage numbers under homeostatic conditions, but it also mediates their rebound after depletion (Ajuebor et al., 1999; Cailhier et al., 2006; Duffield et al., 2005) and inflammation (Ginhoux and Jung, 2014). Hence, the ability of macrophages to proliferate becomes pivotal under inflammatory conditions, which require an enormous increment in macrophage numbers. In a murine model of nematode infection that induces a potent TH2- type immune response, local macrophage proliferation has been described previously (Ajami et al., 2007; Chorro et al., 2009). Proliferation and accumulation were dependent on IL-4 and IL-13 and were restricted to the infection site (Ajami et al., 2007; Hashimoto et al., 2013). Moreover, such IL-4-dependent proliferation was not limited to the resident macrophages population but also stimulated proliferation of monocyte-derived macrophages (Jenkins et al., 2011) indicating that monocytes proliferate even under inflammatory conditions.

However, the role and mechanism of monocyte proliferation under homeostatic and inflammatory conditions is not clear. In addition, the relevance of self-renewal versus recruitment has still not been explored.

1.4 Interleukin 6

Interleukin-6 (IL-6) belongs to a family of cytokines that include LIF (Leukemia inhibitory factor), CT-1 (cardiotrophin-1), IL-11, CNTF (ciliary neurotrophic factor), CLC (cardiotrophin-like cytokine) and OSM (oncostatin M). These members are involved in activation of a variety of genes involved in inflammation, differentiation, survival, proliferation and apoptosis (Davies et al., 2013a; Robbins et al., 2013). Hence, IL-6 family members can act either as pro-inflammatory or anti-inflammatory in different conditions. IL-6 family members are known to interact with glycoprotein 130 (gp130) for their signaling. IL-6 and IL-11 signal via gp130 homo-dimerization, while the other members signal via gp130 heterodimerisation (Davies et al., 2013b).

IL-6 is a pleiotropic cytokine that is known for its ability to promote expansion and activation of T cells and differentiation of B cells (Hashimoto et al., 2013), but also has hormone like functions such as regulating lipid metabolism and insulin resistance (Davies et al., 2011). The role of IL-6 has been extensively studied in several types of tumors and cancers including breast cancer, lung carcinoma, myeloid leukemia, prostrate cancer and ovarian carcinoma (Jenkins et al., 2011). IL-6 is also essential for the switch from innate immunity to adaptive immunity. Therefore, IL-6 plays a major role in shaping our body's immune response (Jenkins et al., 2013).

IL-6 Receptor and signalling

The IL-6 receptor consists of membrane-bound IL-6Ra (mIL- 6Ra, CD126) and the ubiquitously expressed gp130 chain (CD130), which initiates IL-6 signal transduction (Jenkins et al., 2011). Functioning of IL-6R requires the formation of IL-6-IL-6R-gp130 complex (Heinrich et al., 2003; Senaldi et al., 1999). Formation of this complex results in activation of JAK-STAT signaling and the subsequent transcription of downstream effector genes (Heinrich et

al., 2003). IL-6R is expressed mainly on leukocytes, hepatocytes and megakaryocytes, and IL-6R knockout mice are viable. In contrast, gp130 is ubiquitously expressed on all cells and hence its deletion in mice is embryonically lethal (Kishimoto, 2006; Nishimoto and Kishimoto, 2006; Yasukawa et al., 1987). A soluble form of IL-6Ra (sIL-6Ra) exists, because enzymes such as Adam17 and Adam10 actively shed this receptor from IL-6Ra-expressing cells. This sIL-6Ra binds to IL-6 and subsequently interacts with membrane-bound gp130, thereby provoking what is known as trans-signaling (Bethin et al., 2000; Kraakman et al., 2015) (Figure 4). Whereas only few cells express the IL-6 receptor and respond to IL-6 (referred as classical signaling), all cells can be stimulated via a soluble IL-6 receptor – IL-6 complex (trans-signaling), because of ubiquitous expression of gp130 (Bataille et al., 2003; Colomiere et al., 2009; Lee et al., 2003; Sansone et al., 2007; Trikha et al., 2003; Weidle et al., 2010). This widens the IL-6 responsive cells and the scope of IL-6 signaling. It has been shown that in neutrophils, C-reactive protein and other lipid mediators can induce shedding of the IL-6R in response to various stimuli (Jones, 2005). While IL-6 classical signaling is known to regulate homeostatic processes and metabolic pathways (such as glucose metabolism), IL-6 trans signaling is important for inflammatory processes such as recruitment of leukocytes, activation of immune cells and maintenance of T cell effector functions (Chalaris et al., 2011).

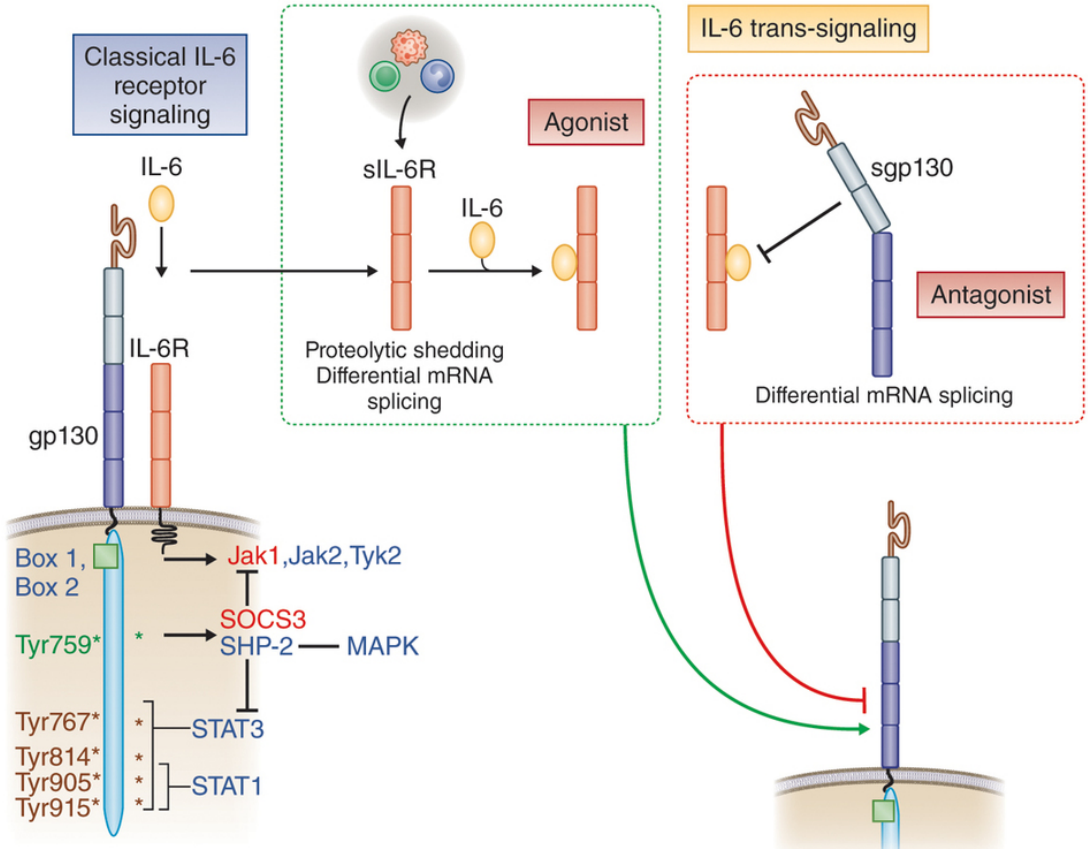

Figure 4: IL-6 signalling pathway.
The two modes of IL-6 signaling pathway (Left) IL-6 binds to membrane bound IL-6R to activate IL-6 classical signalling. (Right) soluble IL-6R (sIL-6R) binds to IL-6 to activate trans-signalling (Skiniotis et al., 2005).

IL-6 in inflammation

IL-6 is a central regulator of inflammatory processes and regulates leukocyte responses following infections (Hunter and Jones, 2015). It has been shown to be vital in the pathogenesis of infection, cancer and inflammation and in the maintenance of immunological reactions (Hunter and Jones, 2015). Impaired IL-6 function causes enhanced susceptibility of mice to infections with various pathogens (Rose-John and Heinrich, 1994). IL-6 has also been shown to be involved in Kaposi's sarcoma as kaposi's sarcoma-associated herpes virus (KSHV) is able to code for a viral homolog of IL-6 (vIL-6) that mimics the human IL-6 and stimulates gp130 thus activating the JAK-STAT signaling to transcribe genes for its own survival (Scheller et al., 2011). Furthermore, vIL-6 has been shown to block infiltration of neutrophils during acute inflammation (Hurst et al., 2002; Jones et al., 1999; Marin et al., 2002).

IL-6 serves as an important target in clinics, for treatment against chronic inflammatory diseases (Hunter and Jones, 2015). It is commonly used as ‘biologic’ (biological response modifier) in many chronic inflammatory diseases with high efficacy such as in rheumatoid arthritis and in Crohn’s disease. Due to elevated levels of IL-6 in sera of patients with different kinds of malignancies, it can also be used as a prognostic marker in a variety of cancers (Hunter and Jones, 2015). While IL-6 blockade has proven to be an effective therapy in some diseases, it has no impact on others. It is unclear why this happens and the role of IL-6 in pathogenesis of these diseases needs to be further studied. The multi functional nature of this cytokine and its ability to signal via classical and trans-signaling leads to complications. Most of the available IL-6 blockage-based therapies target both classical and trans-signaling. Finding specific blockers of trans-signaling could principally remove additional side effects of blocking classical signaling, which is a major player in homeostatic processes.

2. Materials and Methods:

2.1 Materials

Reagents

Item	Manufacturer
BrdU	BD Bioscience
BSA	PAA
CFSE	Life Technologies
D(+)-Saccharose	Carl Roth GmbH & Co KG
FCS	PAA Laboratories
Forene	AbbVie Deutschland GmbH & Co KG
Golgi Plug	BD Bioscience
Immun-Mount	Thermo Scientific
Ketamine 10%	Bela Pharm
L-Lysine Monohydrat	Sigma Aldrich
LB Medium	Carl Roth GmbH & Co KG
N-Hexan	Carl Roth GmbH & Co KG
$Na_2B_4O_7$	Sigma Aldrich
Na_2HPO_4	Carl Roth GmbH & Co KG
NaH_2PO_4	Sigma Aldrich
$NaIO_4$	Carl Roth GmbH & Co KG
PFA	Sigma Aldrich
RPMI 1640 Medium	GE Healthcare
Triton X 100	Carl Roth GmbH & Co KG
Xylazine 2%	CEVA
HEPES	Roth
Heparin-Natrium	Ratiopharm

Table 1: List of Reagents

Buffers

Buffer	Composition
4 % PFA	Dissolved in PBS
L-Lysine Buffer	6.59 g L-Lysine Monohydrate 200 ml P-Buffer
30 % Sucrose	60 g D (+)- Saccharose 200 ml P-Buffer
0.2 M NaH_2PO_4	24 g/l in dH_2O
0.2 M Na_2HPO_4	28.39 g/l in dH_2O
Wash Buffer (PBT)	PBS + 0.05 % Triton X 100
Blocking Buffer	PBT + 1 % BSA
Neutralization Buffer	ddH_2O + 0.1 M $Na_2B_4O_7$
P-Buffer (for 600 ml)	243 ml 0.2 M Na_2HPO_4 57 ml 0.2 M NaH_2PO_4 300 ml autoclaved dH_2O
PLP-Buffer (for 10 ml)	2.5 ml 4 % PFA 3.75 ml L-Lysine Buffer 3.75 ml P-Buffer 0,0212 g $NaIO_4$
FACS buffer (1l)	1l PBS 0.1% FCS 0.1% NaN_3

Table 2: List of Buffers

Materials

Item	**Manufacturer**
Catheter	BD Neoflon
Cover glass	LLG Labware/oehmen Labortechnik

CPS ID plates	Biomerieux
Hydrochloric Acid	Carl Roth GmbH & Co KG
Hydrophobic Pen	Dako Denmark AIS
Immuno Mount	Thermo Fisher Scientific
Instilla gel	FARCO PHARMA
Microscope Slides	R. Langenbrinck Labor & Medizintechnik
Tissue Dispomolds	Formafix
Tissue Tek	Sakura

Table 3: List of Materials

Antibodies and Dyes

Antibody/ Target	Fluoro-chrome	Clone
Rat anti-BrdU		BU1/75 (ICR1)
Goat anti-rat	AF647	Polyclonal
anti mouse Gr1	AF488	RB6-8C5
	FITC	RB6-8C5
	PE	RB6-8C5
	PerCP/Cy5.5	RB6-8C5
anti mouse F4/80	PE	BM8.1
	APC	BM8.1
	BV605	BM8
	BV785	BM8
anti mouse GFP	AF488	Polyclonal
anti mouse Ly6G	FITC	1A8
	BV421	1A8
	AF647	1A8
	Purified	1A8

Nuclei Dyes	DAPI	
	Syto40	
anti mouse CD45	BV421	30-F11
anti mouse CD115	PE	AF-S98
anti mouse Ly6C	Per CP Cy5.5	HK1.4
anti mouse IL-6	Purified	MP5-20F3
Recombinant mouse IL-6	Purified	
anti mouse gp130	Purified	
Recombinant mouse gp130 Fc chimera protein, CF	Purified	
anti mouse IL-4	Purified	11B11
anti mouse IL-10	APC	JES5-16E3

Table 4: List of antibodies and Dyes

Kits

Kit	Company name
BrdU Flow kit	BD Biosciences
IL-6 ELISA kit	R&D Systems
LEGENDplex kit	eBiosciences

Table 5: List of Kits

Instruments

Manufacturer	Name/Technical Details
IMCES Workstation	Computer workstation offering various software
Leica	Cryostat
Leica	SP8 gSTED super-resolution confocal and FLIM
Leica	DM IL LED Fluo

LSR Fortessa™	BD Biosciences
Olympus	BX51 Upright Fluorescence Microscope
Zeiss	AxioObserver.Z1 Inverted Microscope with ApoTome Optical Sectioning and a Live Cell Imaging System

Table 6: List of instruments

Mice

All mice were bred and kept under SPF conditions in the central animal facility in Essen University Clinic. CCR2 -/- mice had been backcrossed >10 times to the C57BL/6 background. C57BL/6 female mice between 8-10 weeks old were purchased from Janvier Laboratories (unless otherwise stated). Animal experiments had been approved by a local animal ethics reviewing board. LysM-*Cre**IL-6R*flox* animals were kindly provided by Hans-Willi Mittrücker, University Hospital, Hamburg. MyD88TRIF -/- and 3D245TLR -/- mice were kind gift from Prof. Carsten Kirschning.

2.2 Methods

Urinary Tract Infection Model

Uropathogenic E. coli strain 536 (O6:K15:H31) were cultured overnight at 37°C in LB medium (LB broth by Roth). Bacteria were harvested by centrifugation at 1200g for 10 minutes and resuspended in 1ml of PBS. Female mice between 8 to 10 weeks of age were anesthetized with a 1:1 mixture of Xylazine 2% (CEVA) and Ketamine 10% (bela Pharm) and infected directly in the bladder using a soft polyethylene catheter (BD Biosciences). The bacterial load was quantified by scoring CFU (CPS ID plates from Biomerieux) after overnight culture of bladder digests at 37°C. For induction of Pyelonephritis, the mice were given a second infection, 3 hrs post the first infection. This second infection results in ascension of UPEC in to the kidneys.

Topical application of antibodies for blocking experiment was done 3 hrs post

infection. Mice were anesthetized and using the same soft catheter as for UPEC infection, antibody mixture was transurethrally injected into the bladder.

Murine peritonitis model

LPS was injected directly into the peritoneum of the mice (i.p). At the time point of sacrifice, 2ml of chilled PBS with 2mM EDTA was injected i.p. Using a syringe, the injected PBS was flushed out. This PBS thus now contains single cell suspension of peritoneal cells. This process was repeated a few times to collect as many peritoneal cells as possible.

Isolation of Leukocytes

A previously described protocol was used. In brief, bladders were sliced with a scalpel into small pieces and digested for 30 minutes at 37°C with 0.5mg/ml collagenase and 100µg/ml DNAse I in RPMI 1640 Medium (GE Healthcare) containing 10% heat-inactivated FCS (PAA Laboratories), 20mM HEPES (Roth), 0.1% ß-mercaptoethanol, 1mM L-Glutamine and antibiotics. Single-cell suspensions were filtered through a 100µm nylon mesh and washed with staining buffer containing 0.1% FCS and 0.1% NaN_3 for surface staining and without NaN_3 for intracellular staining.
Whole blood was obtained from the mice in Heparin-Natrium (Ratiopharm) to prevent clotting and subjected to red cell lysis buffer and washed twice in staining buffer.
Bone Marrow (BM) cells were obtained by flushing the femurs with staining buffer without NaN_3 for in vitro cell culture and with NaN_3 for Flow cytometry.

In vitro culture

Bone marrow cells were obtained by flushing the femurs with PBS and stained with CFSE. CFSE-labeled cells were then cultured in RPMI medium (10% FCS+ P/S+ L-Glu+ 0.5% ß-mercaptoethanol) at 37°C in a 96-well plate with or without stimulants ($3x10^5$ cells/well). 3 days after incubation, cells were detached using 2mM EDTA and single cell suspensions were analyzed by flow cytometry.

CFSE staining

Single cell suspension was incubated with 1mM CFSE dye for 10 minutes at 37°C. The reaction was stopped using chilled medium and the cells were washed twice. The cells were recounted again after CFSE labelling and appropriate amount of cells were then used for in vitro culture.

Flow Cytometry

Single-cell suspensions were incubated with mouse serum to block Fc receptors. Titrated amount of the Fluorochrome labeled antibodies were used for staining of 1 * 10^6 cells per sample.

For intracellular staining, the surface stained samples were fixed with 4% PFA and 1µl/ml Golgi Plug (BD) was used for 4 hours to block cellular release of chemokine and washed with Perm wash buffer (BD) instead of staining buffer. Cells were measured on LSR Fortesa (BD) and analyzed using Flow Jo software (Tristar).

Absolute cell numbers were calculated by adding APC labeled microbeads (BD) to each sample.

BrdU Incorporation Assays

Surface stained samples were processed for BrdU staining using the protocol as stated in the BrdU Kit (FITC BrdU Flow Kit by BD Pharmingen). In brief, surface stained single cell suspension was fixed using BD Cytofix/Cytoperm Buffer for 10 minutes. Samples were next fixed BD Cytoperm buffer plus for another 10 minutes and washed using perm wash buffer. Samples were then treated with DNAse (provided in the kit) for 1 hour at 37°C. DNAse treated samples were then stained with anti BrdU antibody (provided in the kit) for 30 minutes at RT followed by washing step with per wash buffer. The stained samples were then ready to be measured by a flow cytometer.

IL6 ELISA

Bladders were homogenized in the presence of proteinase inhibitors (Complete; Mini EDTA free from ROCHE) in 500µl PBS. Homogenates were

centrifuged at 12000 rpm for 20 minutes and supernatants were stored at -80°C. Blood plasma was obtained using Heparin-Natrium and centrifuging it at 2000 rpm for 20 minutes. Both bladder supernatants and blood plasma were analysed using Quantikine anti mouse IL6 ELISA Kit (R and D systems)

Cytokine Array

Bladders were homogenized in the presence of proteinase inhibitors (Complete; Mini EDTA free from ROCHE) in 500µl PBS. Homogenates were centrifuged at 12000 rpm for 20 minutes and supernatant was directly used for the measurement using the standard protocol provided with MILLIPLEX MAP Mouse Cytokine/Chemokine Magnetic Bead Panel -Multiplex Assay (Merck Millipore).

Histology

Bladders were fixed in PLP buffer adjusted to 7.4 pH (4% PFA, NaIo4, L-Lysine, P-buffer) overnight followed by treatment with 30% sucrose overnight. The bladders were then embedded in Tissue-Tek® OCT in the presence of dry ice and n-hexan and stored at -80°C. The sectioning were performed at -20°C with a Cryostat. The sections (10µm) were stained with DAPI (1:5000), F4/80 PE (1:200), Gr1 AF488 (1:200), rat anti mouse BrdU (1:500), anti rat AF647 (1:500), measured using Zeiss AxioObserver.Z1 and Apotome and analyzed using ImageJ.

	Treatment	Amount	Time	Temperature
1	Pre-warm heating plate			70°C
2	Fill 1N HCL into a plastic chamber and pre-warm it in the water bath			37°C
3	Fixation of the slides		5 - 10min	70°C (Heating plate)
4	Denaturation of DNA with 1N HCL		30min	37°C
5	Neutralization with $Na_2B_4O_7$, pH 8.5		2 x 5min	RT
6	Wash with PBT		3 x 5min	RT
7	Blocking in 1% BSA-PBT	500µl/	1h	RT

		slide		
8	Dry the edges of the slides with a tissue (carefully!)			
9	Draw a border with the hydrophobic pen, let it dry		5 min	RT
10	Dilute rat anti-BrdU (1:500) in 1% BSA-PBT	200µl/ slide	Over-night or 2h	4°C or RT
11	Wash with PBT		3 x 5min	RT
12	Dilute goat anti-rat AF647 (1:500) in 1% BSA-PBT	200µl/ slide	1h	RT
13	Wash with PBT		3 x 5min	RT
14	Dilute F4/80 PE (1:200) in 1% BSA-PBT	200µl/ slide	1h	RT
15	Wash with PBT		3 x 5min	RT
16	Dilute DAPI (1:5000) in 1% BSA-PBT	200µl/ slide	5min	RT
17	Wash the slides with Bi-dest water		Few sec.	
18	Knock off the liquid			
19	Mount with Immuno-Mount			
20	Dry		Over-night	RT, cover from light

Table 7: Step by step protocol for immunofluorescence staining of bladder sections.

Image processing

Image J software was used to process all the images that were acquired with the microscope. Borders were made around the region of interest (For example: epithelium) to generate distance map. These distance maps were used to calculate distance of each cell of our interest from the border (For example: from the epithelium).

ImageJ was also used to generate masks for each channel. These masks were then overlapped to generate a single point. Each point thus represented the combined fluorescent signal being expressed at that region. A point map of proliferating Gr1+ monocytes thus represents points in the entire bladder section where all three (F4/80+Gr1+BrdU+) of the fluorescent signals were expressed.

	Steps
1	Duplicate DAPI or GFP channel
2	Press “t” to open the ROI Manager
3	Use “Polygon selection” → draw a border to define the epithelium → press “t” to add it into ROI → draw another border if needed and press “t” again
4	Click on ROI and select all defined borders by “Ctrl+a”
5	ROI → More → OR (Combine)
6	Edit → Clear outside
7	Edit → Fill (Lumen should be of a significantly different color (e.g. blue)
8	Select all “islands” in ROI, meaning all borders that are within a defined area (1 and 2 in the example above)
9	ROI → More → OR (Combine)
10	Edit → Clear
11	Image → Type → Convert it into 8-bit
12	Process → Binary → Convert to Mask → wait until its black and white
13	Edit → Invert (the area you are interested in should be white)
14	“s” save as tiff
15	Process → Binary → Options → Pad edges: ON, EDM: 16-bit
16	Process → Binary → Distance Map → “s” save as tiff

Table 8: Step by step protocol for making border and distance map on an acquired image.

	Steps

1	Duplicate DAPI
2	Process → Filters → Gaussian Blur → ~2 (remove any local variations within the nucleus + fill in the gaps)
3	Image → Type → 8-bit
4	Image → Adjust → Auto local threshold → Method: Bernsen, Radius: 10 (roughly the order of the size of the nucleus, just that you are really looking locally)
5	Process → Binary → Watershed (to separate cells/ nuclei)
6	Analyze →Analyze Particles: Size: 10-Infinity; Circularity: 0-1; Overly Masks; Add into ROI manager → Image → Overlay → remove overlay → On Image ctrl+A → Edit → Clear → On ROI Data ctrl+A →More → Fill
7	Process → Image Calculator "Nuclei-Mask" AND (OPERATION) "DAPI-Mask"
8	Save as Nuclei Mask

Table 9: Step by step protocol for making a nuclei mask.

	Steps
1	Duplicate Gr1 Channel
2	Open/Clear ROI manager
3	Process → Filters → Gaussian Blur ~2
4	Convert into 8bit
5	Image → Adjust → Threshold (RED) → Apply
6	Process → Binary → Watershed
7	Analyse →Analyse Particles: Size: 0-250; Circularity: 0.2-1; Overly Masks; Add to ROI manager → Image → Overlay → remove overlay → On Image ctr+A → Edit → Clear → On ROI Data ctr+A →More → Fill

8	Save as “Gr1 Mask” or “F4/80 Mask” or “BrdU Mask”

Table 10: Step by step protocol for generating masks on Gr1 fluorescence channel.

	Steps
1	Duplicate Gr1 Channel (or F4/80 or BrdU)
2	Open/Clear ROI manager
3	Process → Filters → Gaussian Blur ~2
4	Convert into 8bit
5	Image → Adjust → Threshold (RED) → Apply
6	Process → Binary → Watershed
7	Analyse →Analyse Particles: Size: 0-250; Circularity: 0.2-1; Overly Masks; Add to ROI manager → Image → Overlay → remove overlay → On Image ctr+A → Edit → Clear → On ROI Data ctr+A →More → Fill
8	Save as “Gr1 Mask” or “F4/80 Mask” or “BrdU Mask”

Table 11: Step by step protocol for generating masks on Gr1 fluorescence channel.

	Steps
1	Duplicate F4/80 channel
2	Open/Clear ROI manager
3	Process → Filters → Gaussian Blur ~2
4	Convert into 8bit
5	Image → Adjust → Threshold (RED) → Apply
6	Save as “F4/80 Mask”

Table 12: Step by step protocol for generating masks on F4/80 fluorescence channel.

	Steps
1	Duplicate BrdU channel
2	Open/Clear ROI manager
3	Use threshold (RED) to choose signals → apply
4	Save as "BrdU Mask"

Table 13: Step by step protocol for generating masks on BrdU fluorescence channel.

	Steps
1	Open the nuclei, F4/80 and BrdU mask
2	Generate the BrdU+/- masks (as described above)
3	Save masks
4	Combine the masks using the Image calculator and AND operation
5	Save as 'Gr1+F4/80+BrdU+'
6	Save as 'Gr1+F4/80+BrdU-'

Table 14: Step by step protocol for generating masks on Gr1+F4/80+BrdU+/- fluorescence channel.

Statistical Analysis

Data was analyzed using Prism software (GraphPad) and Mann-Whitney test was used for significance analysis. In all figures, * indicates p-value >0.05, ** >0.01 and *** >0.001

3. Results

3.1.1 Histological analysis of uninfected mouse bladders

Uninfected bladders of C57BL/6 mice were analyzed by histology by staining with F4/80, Gr1, BrdU and DAPI. F4/80 was stained to visualize macrophages, Gr1 to visualize inflammatory monocytes and neutrophils and lastly, BrdU to visualize proliferating cells. There was presence of F4/80+ macrophages in a homeostatic bladder. However, there were almost no inflammatory monocytes (DAPI+ F4/80+ Gr1+) and neutrophils (DAPI+ F4/80– Gr1+) in the uninfected bladder. Also, BrdU+ proliferating cells could not be detected (**Figure 5A**).

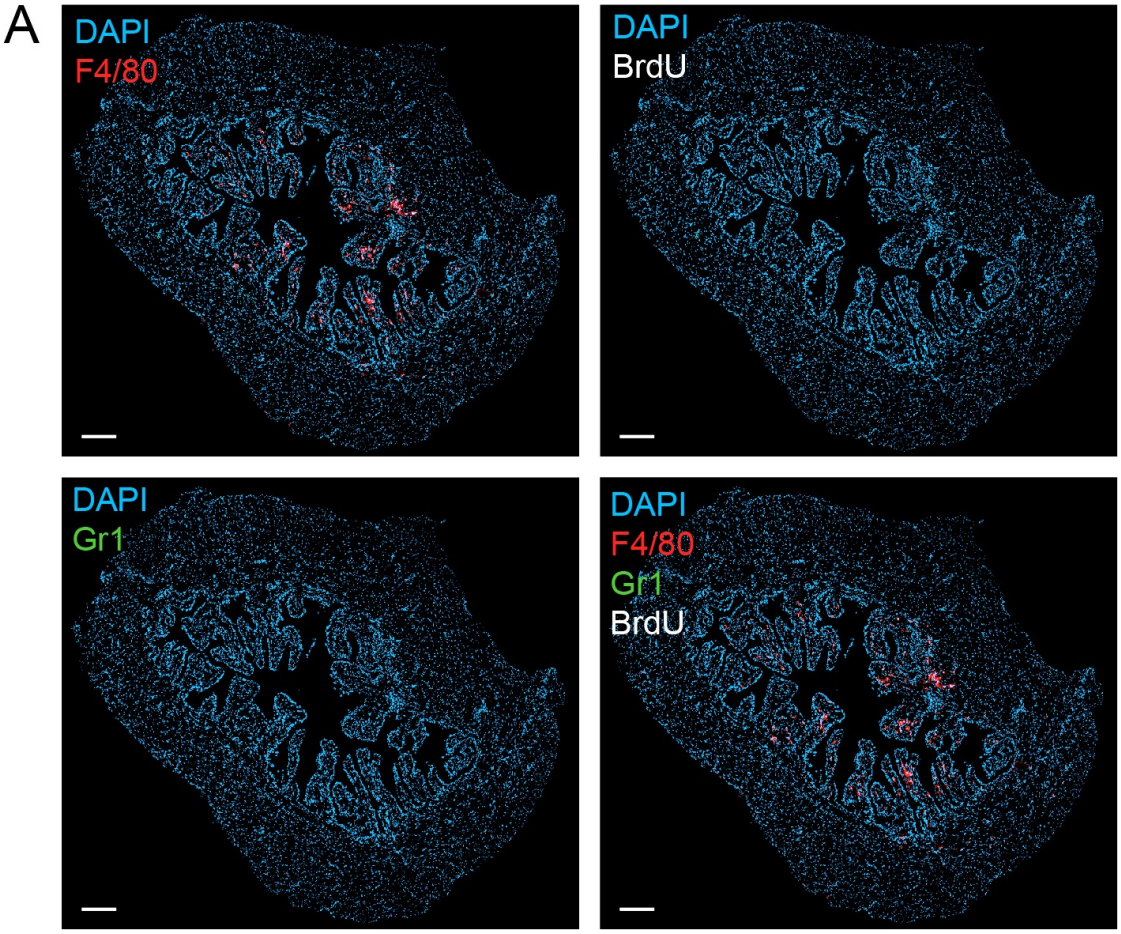

Figure 5: Histological analysis of uninfected mouse bladders.
Bladders of C57BL/6 mice were isolated and processed for histology. BrdU was injected in the peritoneum of the mice 24 hrs before sacrifice. (A) Bladder sections were stained for DAPI (blue) F4/80 (red), BrdU (white) and Gr1 (green). The white scale bar indicates 200µm.

3.1.2 F4/80+ macrophages proliferate in the infected urinary bladder

There is emerging evidence from recent studies in mice that tissue macrophages can be rescued from cell cycle arrest (Chalaris et al., 2011). We tested this in in a model of bacterial urinary bladder infection induced by uropathogenic *E. coli* (UPEC). Bladder sections were stained for DAPI (nucleus), F4/80 macrophages and BrdU (proliferating cells). We detected proliferating BrdU+F4/80+ cells in the infected urinary bladder by microscopy (**Figure 6A**). These data indicated that macrophages in infected bladders might also have the capability to overcome cell cycle arrest.

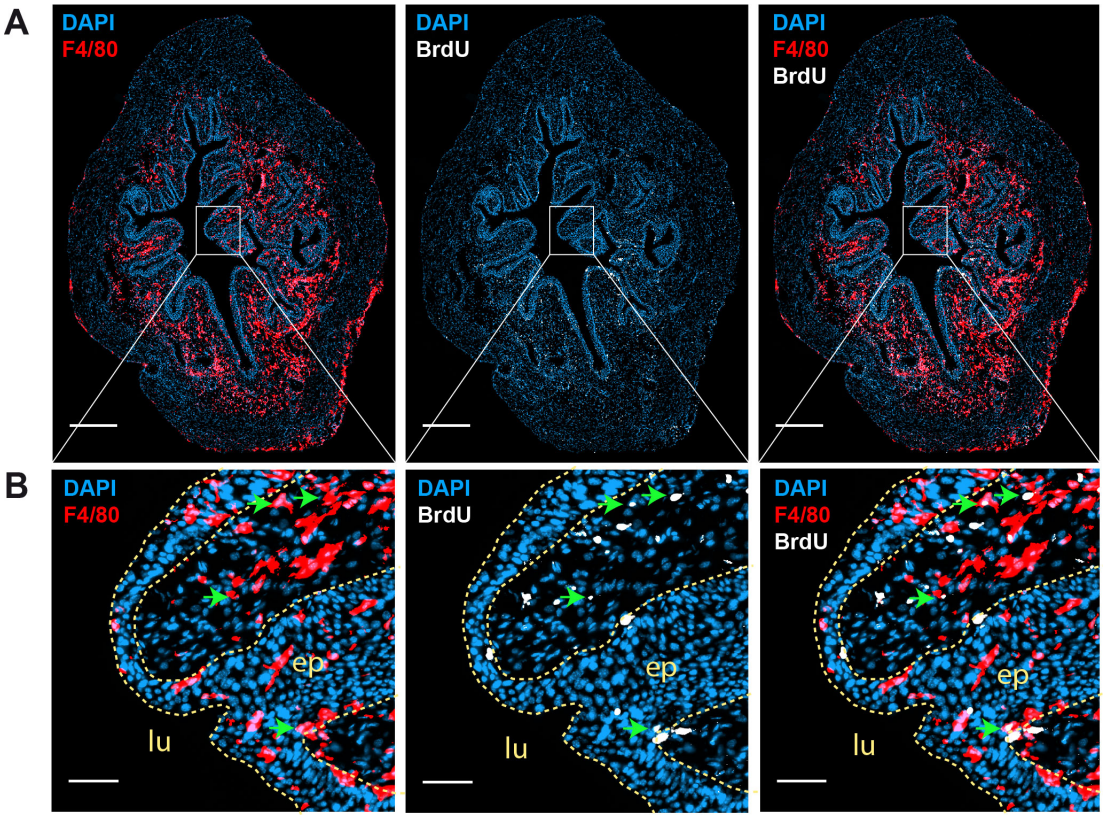

Figure 6: F4/80+ macrophages proliferate in the infected urinary bladder.
C57BL/6 mice were infected transurethrally with uropathogenic *E.coli* (UPEC). BrdU was administered into the peritoneum of mice at the time of infection. (A) Bladder sections were stained for DAPI (blue), F4/80 (red), and BrdU (white) one day after infection. The white scale bar indicates 50µm. (B) The green arrow in the detailed images indicates proliferating macrophages (DAPI+F4/80+BrdU+). The white scale bar indicates 200µm. Data are representative of two experiments (mean ± SEM, n = 5). ep=Epithelium; lu=Lumen.

3.1.3 Ly6C+ monocytes proliferate in the infected urinary bladder

The presence of proliferative F4/80+ macrophages was also confirmed by flow cytometry (**Figure 7A**). Most of these F4/80+ cells also expressed Ly6C and CD64 on their surface (**Figure 7B**) demonstrating that these cells originated from recruited Ly6C+ blood monocytes. Specific staining of Ly6C on F4/80+ cells revealed vigorous proliferation of Ly6C+ monocytes after infection and these proliferating cells accumulated during the course of infection (**Figure 7C and D**). Notably, the number of non-proliferating (BrdU–) Ly6C+ monocytes increased until day 1, but rapidly declined during the course of the disease (**Figure 7C**). These findings demonstrate proliferation of Ly6C+ monocytes in urinary tract infection and extend the current model of local tissue macrophage proliferation to recruited Ly6C+ monocytes.

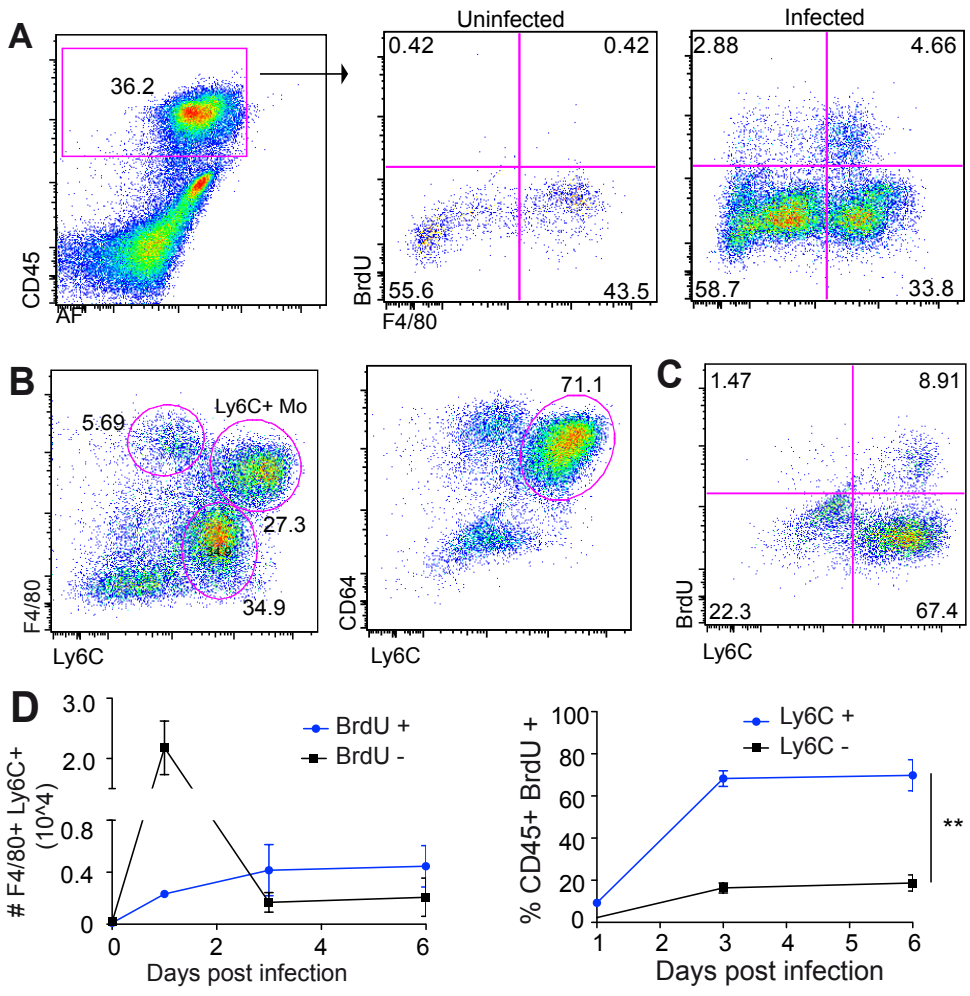

Figure 7: Ly6C+ monocytes proliferate in the infected urinary bladder.

C57BL/6 mice were infected transurethrally with uropathogenic *E.coli* (UPEC). BrdU was administered into the peritoneum of mice at the time of infection. Bladders were analyzed 24 hrs post infection. (A) Incorporation of BrdU by CD45+F4/80+ cells one day after infection. (B) One day after infection, the expression of F4/80, Ly6C and CD64 was analyzed on CD45+ leukocytes (left cytogram) and on CD45+F4/80+ cells (right cytogram). (C) Incorporation of BrdU by CD45+F4/80+Ly6C+ monocytes one day after infection and (D) at various time points after infection (longitudinal analysis). Data are representative for two experiments (mean ± SEM, n = 5).

3.1.4 Proliferating Ly6C+ monocytes localize to the lamina propria

Since Ly6C+ monocytes actively proliferate in the infected bladders. It was interesting to determine the localization of these cells within the bladder. Next, the localization of proliferating monocytes versus non-proliferating monocytes was analyzed by histology. One day after inducing the bladder infection, bladder sections were stained with the antibody Gr1 (Ly6C/Ly6G), which detects Ly6C+ monocytes and Ly6C/G+ neutrophils. Additionally, bladder sections were stained with DAPI (nucleus), F4/80 (macrophages) and BrdU (proliferation). Masks were generated for each fluorescent channel (described in methods under Image processing) to directly depict (F4/80+Gr1+BrdU+) proliferating monocytes and (F4/80+Gr1+BrdU-) non-proliferating monocytes on the images. Proliferating monocytes are thus indicated as white quadrants and non-proliferating monocytes as magenta quadrants. Proliferating monocytes were mostly located to the lamina propria (**Figure 8A**, white quadrant in the lower right image). In contrast, neutrophils and non-proliferating monocytes were also numerous within the infected bladder epithelium (**Figure 8A, B**). As the number of neutrophils rapidly declined over the course of the infection (**Figure 8C**), leading to the hypothesis that non-proliferating monocytes might be involved in phagocytosis.

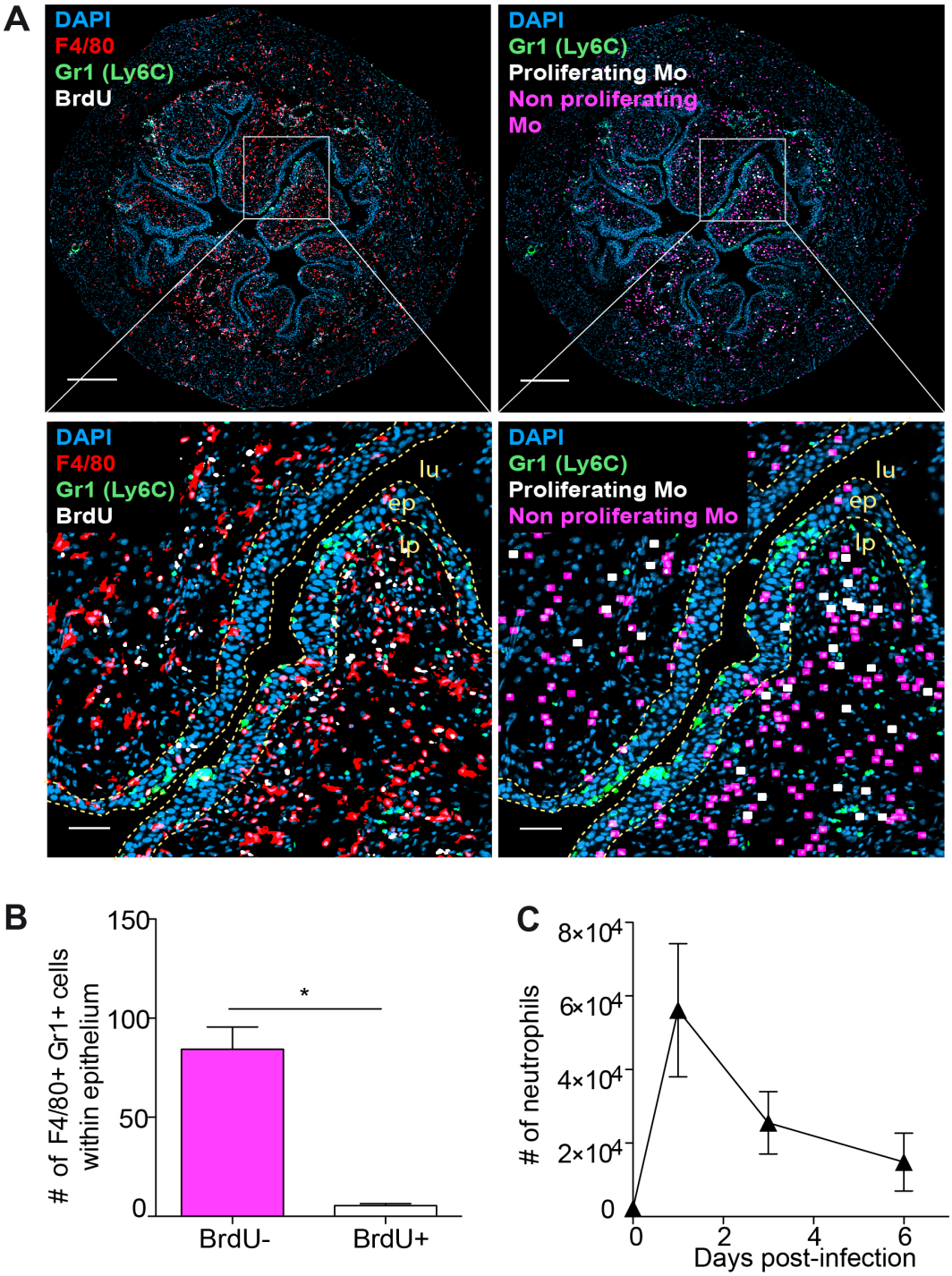

Figure 8: Localization of proliferating monocytes in the infected bladder.

(A) One day after infection, bladder sections were stained for DAPI (blue), F4/80 (red), Gr1 (green) and BrdU (white). The raw microscopy (left images) was further processed to generate point maps (right images). The point maps show the positions of cells with combinations of fluorescent signals for F4/80, Gr1 and BrdU. The white and magenta quadrants indicate proliferating (F4/80+Gr1/Ly6C+BrdU+) and non-proliferating (F4/80+Gr1/Ly6C+BrdU–) monocytes respectively. (B) Quantification of proliferating and non-proliferating monocytes within the epithelium of infected bladders by microscopy (original images in (A). (C) Number of F4/80–Gr1+ neutrophils over the course of infection. The white bar indicates 50µm (top) and 200µm (bottom). Data are mean ± SEM; n=8. ep=epithelium; lp=lamina propria; lu=lumen.

3.1.5 Non Proliferating Ly6C+ monocytes phagocytize neutrophils

To test the hypothesis that non-proliferating monocytes phagocytize matured neutrophils because of their presence close to neutrophil cluster, the neutrophil-specific molecule Ly6G was stained intracellular in F4/80+Ly6C+ monocytes. Indeed, this molecule was detected only in non-proliferating Ly6C+ monocytes (**Figure 9A, B**) indicating that the rapid disappearance of neutrophils during the infection may partially be facilitated by phagocytic non-proliferating monocytes.

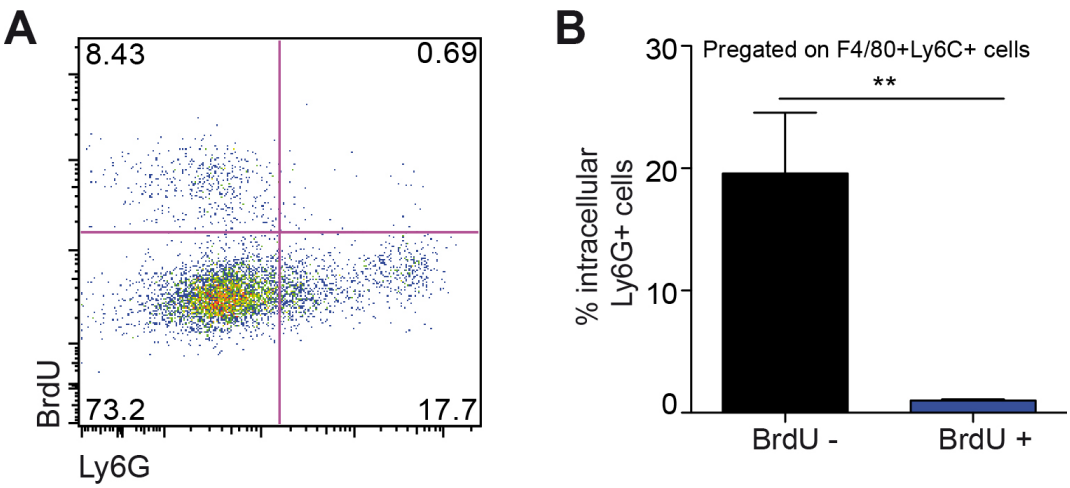

Figure 9: Non-proliferating monocytes phagocytize neutrophils in the infected bladder.
(A) Intracellular detection of Ly6G in CD45+F4/80+Ly6C+ monocytes in infected bladder one day after infection (B) Frequency of CD45+F4/80+Ly6C+ monocytes with intracellular detection of Ly6G one day after infection. Data are mean ± SEM; n=10.

3.1.6 IL-6 induces the proliferation of Ly6C+ monocytes

To further analyze the mechanism of local monocyte proliferation, the inflammatory milieu was determined in the infected urinary bladder by a comprehensive screening assay (**Figure 10A**). IL-6, IL-13, G-CSF and CXCL2 were strongly up regulated one day after infection (**Figure 10A**). As IL-6 was one of the most up regulated proteins and their crucial proinflammatory role has been demonstrated previously, the contribution of IL-6 for monocyte proliferation was further investigated. To this end, BM monocytes were stimulated with IL-6 to determine the capacity of this inflammatory molecule for monocyte proliferation. Supplementing IL-6 strongly

induced the proliferation of Ly6C+ monocytes in a dose-dependent manner (**Figure 10B, C**). Notably, Ly6C– monocytes did not proliferate after supplementation of IL-6 identifying IL-6 as a crucial and specific stimulatory molecule for the proliferation of Ly6C+ monocytes.

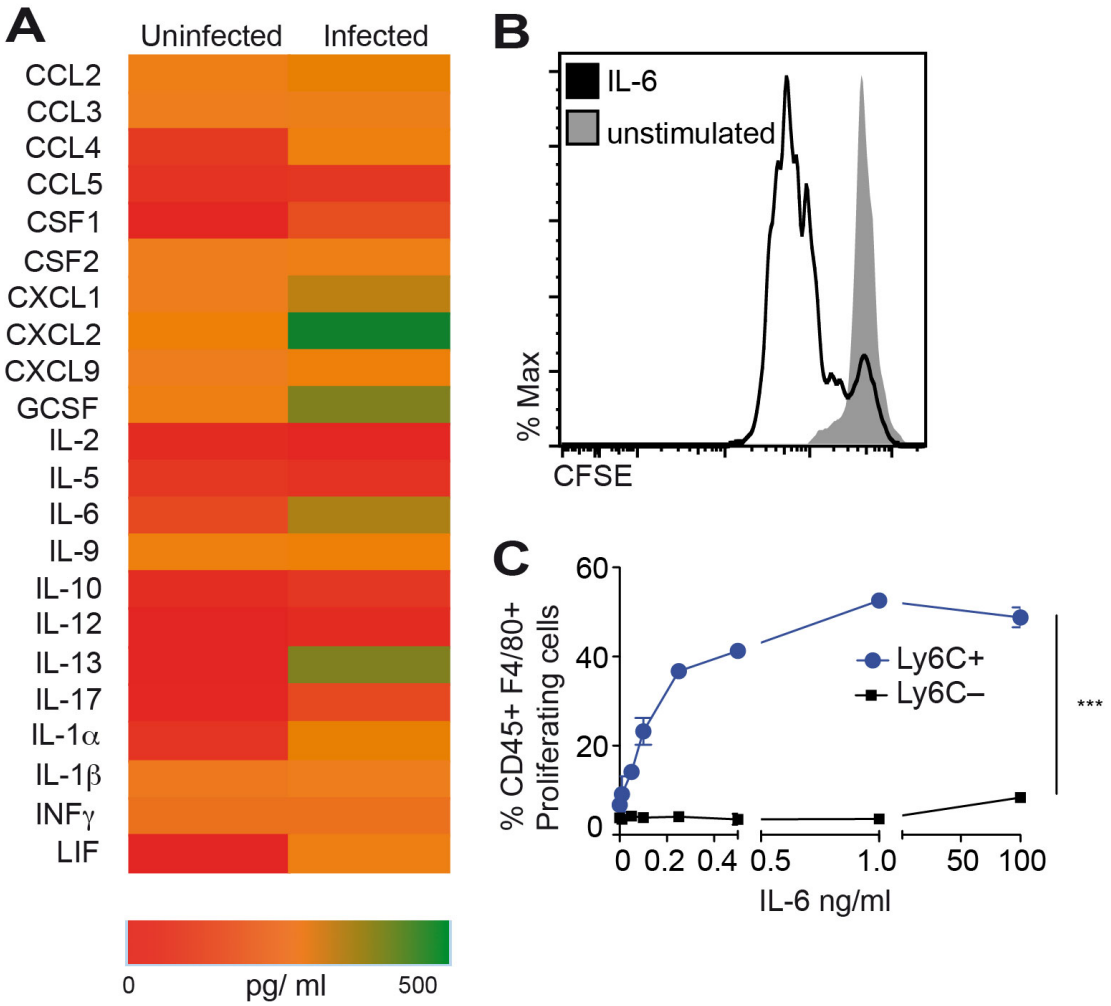

Figure 10: IL-6 induces the proliferation of Ly6C+ monocytes.
(A) C57BL/6 mice were transurethrally infected with UPECs. One day after infection, the levels of the indicated molecules were measured by a Luminex assay and the concentrations of the measured molecules are depicted as a heat map. (B,C) Bone marrow cells from C57BL/6 were stained with CFSE and cultured for 3 days in the presence and absence of IL-6. Proliferation of CD45+F4/80+Ly6C+ monocytes (B) and dose-dependent proliferation of CD45+F4/80+Ly6C– and CD45+F4/80+Ly6C+ monocytes was measured by the CFSE dilution (C). Data are mean ± SEM; (A) n=10, (B,C) n=5.

3.1.7 Blocking IL-6 reduces the proliferation of Ly6C+ monocytes during infection

The stimulatory capacity of IL-6 for the proliferation of BM Ly6C+ monocytes raised the question whether this molecule also drives the proliferation of Ly6C+ monocytes during urinary tract infection. To test this hypothesis, IL-6 was locally inhibited by instillation of an anti-IL-6 antibody into the bladder lumen of infected mice. As the anti IL-6 is a monomeric IgG antibody, it can easily diffuse into the tissues. Administration of this antibody significantly reduced the proliferation of Ly6C+ monocytes (**Figure 11A, B**). In a next experiment, the glycoprotein gp130 was inhibited, the essential molecule for IL-6 signaling. Blocking of gp130 by instilling an anti-gp130 antibody locally into the bladder completely abolished the proliferation of Ly6C+ monocytes demonstrating the essential role of IL-6-signaling in regulating proliferation of Ly6C+ monocytes (**Figure 11A, B**). Notably, proliferation of Ly6C– macrophages was hardly detectable and this low proliferation was unaffected by the IL-6 and the gp130 blockade.

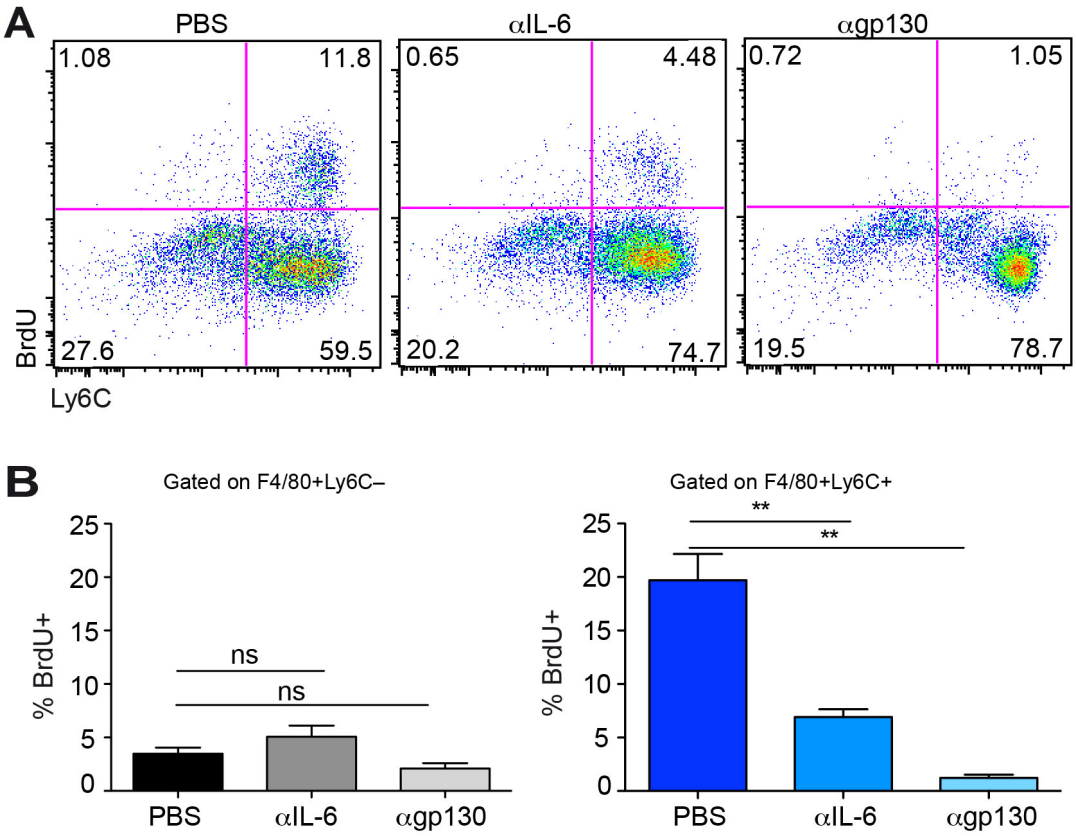

Figure 11: Blocking IL-6 reduces the proliferation of Ly6C+ monocytes.
C57BL/6 mice were transurethrally infected with UPECs. (A,B) Three hours after infection, an antibody against IL-6, gp130 or Sgp130 was instilled into the bladder and proliferation of CD45+F4/80+ monocytes were analyzed one day after infection. (B) Quantitative analysis of (A). Data are mean ± SEM; n=8.

3.1.8 IL-6 regulates the proliferation of Ly6C+ monocytes during peritonitis

Next, the proliferation of Ly6C+ monocytes in other organs was investigated. To see whether proliferation of Ly6C+ monocytes also exists in other inflammatory settings. To this end, LPS was injected into the peritoneum of mice to mimic an acute bacterial infection. We found a significantly increased frequency of Ly6C+ macrophages one day after injection (**Figure 12A**). These Ly6C+ macrophages strongly incorporated BrdU demonstrating vigorous proliferation (**Figure 12B**), whereas Ly6C– macrophages hardly incorporated BrdU (**Figure 12C**). These finding were similar to the findings in the infected bladder. Next step was thus to analyze the role of IL-6 in regulating proliferation of Ly6C+ monocytes in the inflamed peritoneum. Hence, IL-6 was inhibited using an IL-6 antibody that was injected into the peritoneum. Like the

inhibition approach in the urinary bladder described in the previous paragraph, this IL-6 antibody was also an IgG antibody and due to its small size it can easily exerts it´s function in the peritoneum.. Inhibition of IL-6 in this *in vivo* setting also significantly reduced the proliferation of Ly6C+ monocytes (**Figure 12D**). These data collectively demonstrate that proliferation of Ly6C+ monocytes depends on IL-6 in an organ-independent manner.

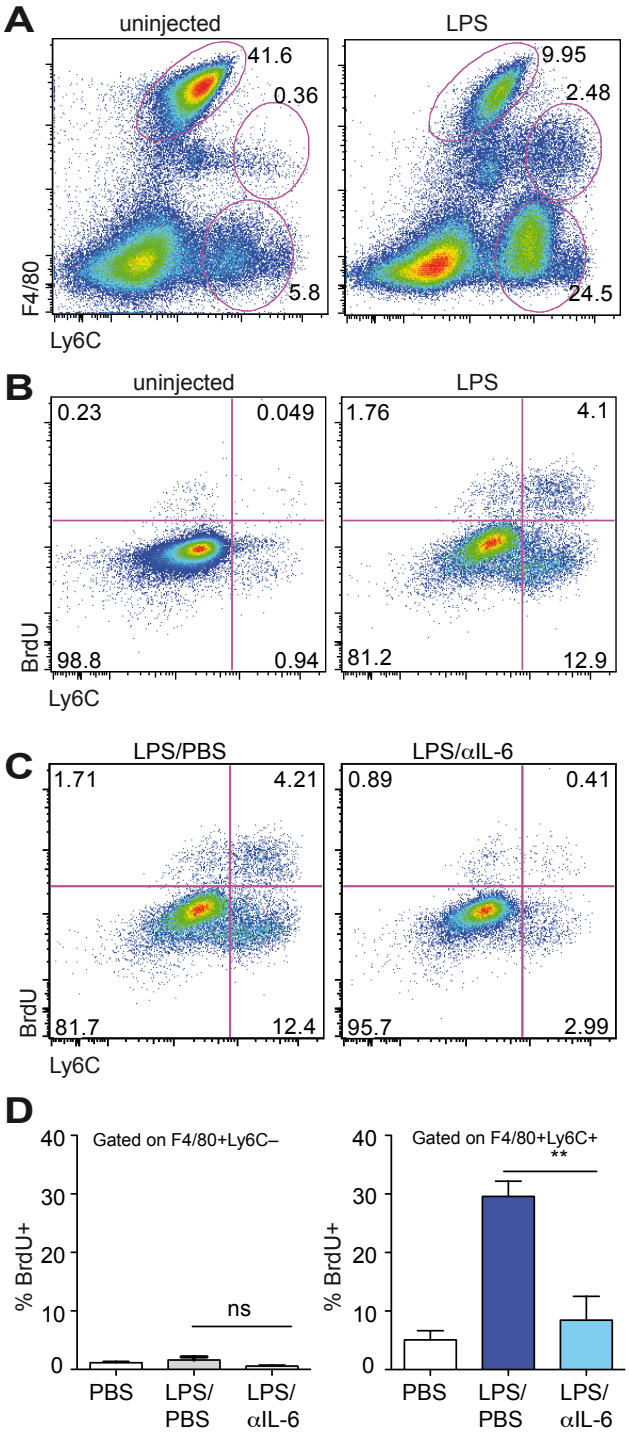

Figure 12: Proliferation of Ly6C+ monocytes in the inflamed peritoneum depends on IL-6.
C57BL/6 mice were injected intraperitoneally with LPS or PBS as a control. Proliferation of F4/80+ monocytes in the peritoneal exudate was analyzed by injection of BrdU intraperitoneally at the time point of LPS/PBS administration. Proliferation was measured one day later. (A) Representative flow cytogram depicting accumulation of CD45+ leukocytes in the peritoneum. (B,C) BrdU incorporation by CD45+F4/80+ monocytes in the peritoneum (B) and in the inflamed peritoneum of mice injected with aIL-6 antibody-treatment or PBS as a control (C). (D) Quantification of (C): Frequency of proliferating F4/80+Ly6C–monocytes (left bar graphs) and F4/80+Ly6C–monocytes (right bar graphs). Data are mean ± SEM; n=8.

3.1.9 Blocking IL-6 trans-signalling reduces the proliferation of Ly6C+ monocytes

Next, whether IL-6 induced proliferation is regulated by IL-6 classical or trans-signalling was investigated. We instilled soluble gp130 (Sgp130), which specifically blocks IL-6 trans-signalling (Hunter and Jones, 2015). Significantly reduced incorporation of BrdU by Ly6C+ monocytes was found in these animals (**Figure 13A, B**) identifying IL-6 trans-signalling as the mechanism that regulates proliferation of Ly6C+ monocytes. Again, this effect was specific for Ly6C+ monocytes and no difference was seen in proliferation of Ly6C- monocytes (**Figure 13B; left graph**).

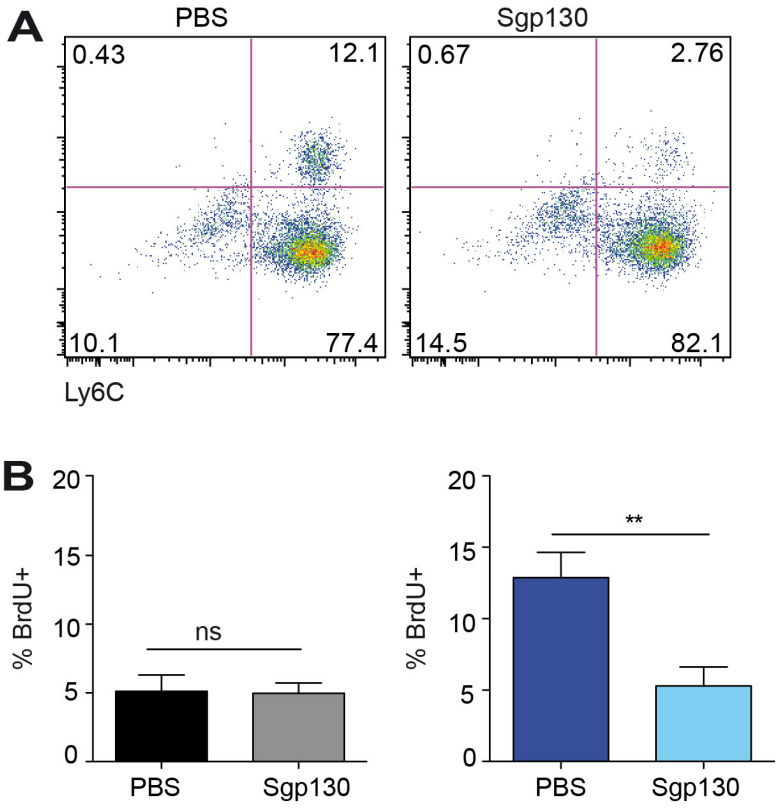

Figure 13: IL-6 trans-signalling induces the proliferation of Ly6C+ monocytes.
(A) C57BL/6 mice were infected with UPECs and analyzed one day after infection. Three hours after infection, Sgp130 was instilled transurethrally to block IL-6 trans-signaling and proliferation on CD45+F4/80+ monocytes was measured by BrdU incorporation. (B) Representative flow cytogram depicting BrdU incorporation by CD45+F4/80+ monocytes. Data are mean ± SEM; n=5

3.1.10 CD45-negative cells produce IL-6 in the infected bladder

To investigate the source of IL-6, the production of this molecule was analyzed by flow cytometry. Flow cytometry analysis revealed that IL-6 was mostly produced by CD45-negative cells. CD45- cells can include epithelial cells, endothelial cells, fibroblasts etc. These data thus indicated that non-hematopoietic cells like epithelial cells, endothelial cells or fibroblasts might secrete this important molecule (**Figure 14A, B**). These data demonstrate the source of IL-6 in infected bladders.

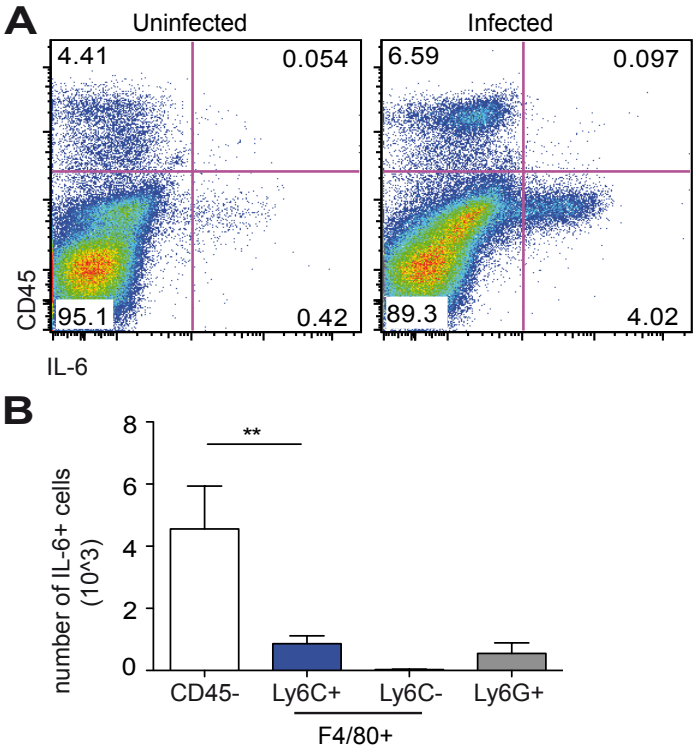

Figure 14: CD45-negative cells produce IL-6 in the infected bladder.
(A) One day after infection, the expression of IL-6 was measured by flow cytometry. Representative flow cytogram depicting intracellular IL-6 expression by CD45+ and CD45– cells in the uninfected and one day after infection. (B) Quantification of (A) for IL-6 expression. Data are mean ± SEM; n=8.

3.1.11 Proliferation of Ly6C+ monocytes is independent of TLR signaling

TLR signalling is an important pathway and crucially involved in the pathogenesis of UPEC infections. To investigate if the proliferation of Ly6C+ monocytes depends on this signalling pathway, we utilized MyD88TRIF double knock out mice. These mice lack the adaptor molecule that is essential for TLR signalling, namely MyD88 and TRIF. Additionally, the mice that lack TLR2, TLR4, TLR5 and 3D TLR signalling (3D245TLR) were also used. Flow cytometry analysis of these mice infected with UPECs revealed, similar frequencies of Ly6C+BrdU+ monocytes in the infected bladders (**Figure 15A and B**) indicating that the trigger for proliferation does not come via TLR signalling cascade.

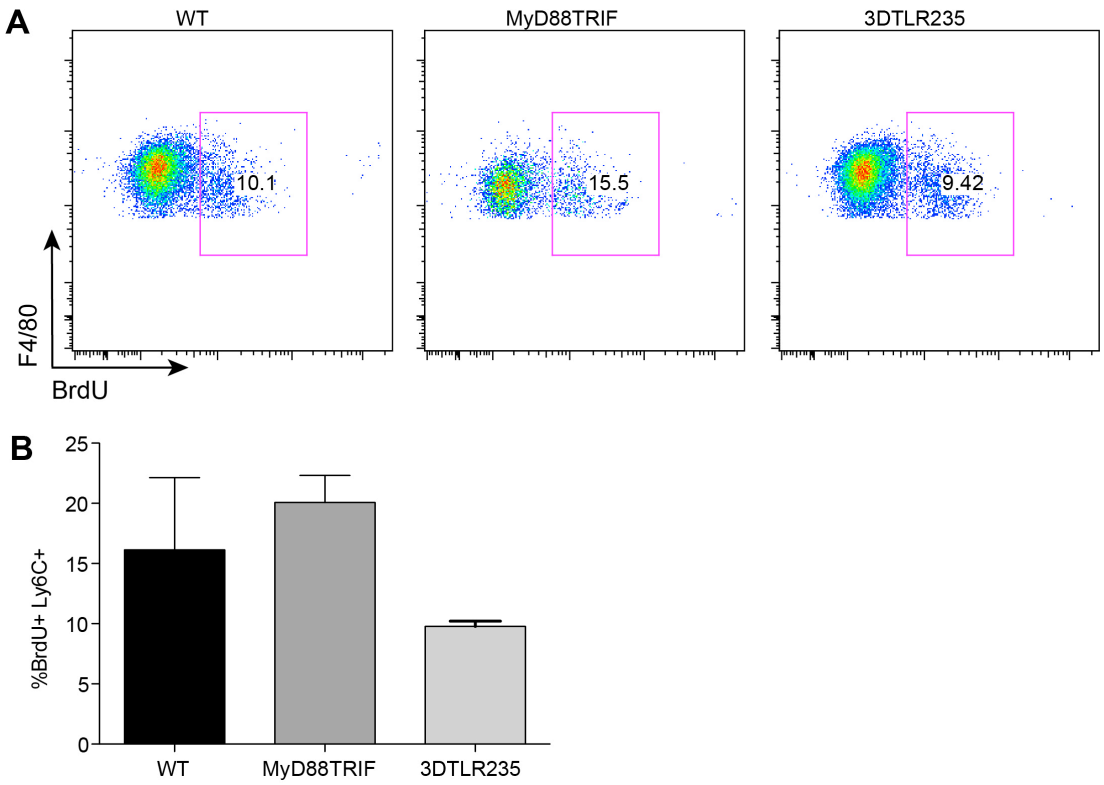

Figure 15: Proliferation of Ly6C+ monocytes is independent of TLR signalling pathway.
WT, MyD88TRIF KO and 3D235TLR KO mice were transurethrally infected with UPECs. One day after infection, the bladders were isolated and analyzed by flow cytometry. (**A**) Flow cytogram depicting proliferating (BrdU+) monocytes. (**B**) Frequencies of Ly6C+BrdU+ cells in the infected bladder of WT, MyD88TRIF KO and 3D235TLR KO mice. Data are mean ± SEM, n=5.

3.1.12 Blocking IL-6 signalling does not effect recruitment.

Monocytes are recruited to the infected tissues where they perform several effector functions (Dann et al., 2008; Diao and Kohanawa, 2005; Dube et al., 2004; Kopf et al., 1994; Ladel et al., 1997; Romani et al., 1996). Next, the impact of blocking IL-6 signalling on recruitment was determined. Blocking IL-6 and gp130 using antibodies did not result in decreased frequency (**Figure 16A**) and reduced number of Ly6C+ monocytes in the infected bladders (**Figure 16B**). These data indicate that the overall number of Ly6C+ monocytes in infected bladders is unaffected by IL-6 blocking and only the proliferative capacity of Ly6C+ monocytes is impaired.

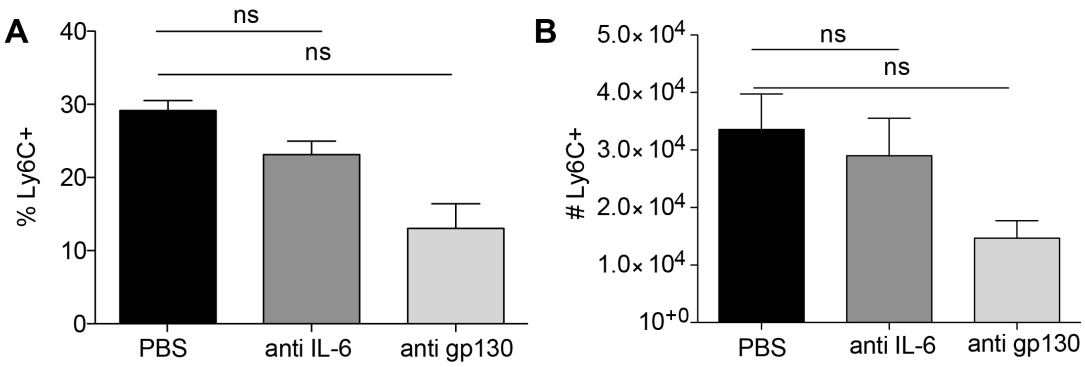

Figure 16: Blocking IL-6 signalling does not impair recruitment of Ly6C+ monocytes.
C57BL/6 mice were infected with UPECs and analyzed one day after infection. (**A,B**) Three hours after infection, PBS, aIL-6 or gp130 was instilled transurethrally to block IL-6 signalling and the frequency of Ly6C+ monocytes (**A**) and their numbers (**B**) was determined by flow cytometry. Data are mean ± SEM, n=8.

3.1.13 Blocking IL-6 trans-signalling aggravates the infection

Next, the role of IL-6-dependent monocyte proliferation for anti-bacterial defense against uropathogenic *E.coli* was determined. Blocking proliferation by administration of Sgp130, anti IL-6 and anti gp130 into the urinary bladder significantly increased the levels of neutrophils (**Figure 17A**). Furthermore, levels of chemokines such as CCL2, CCL3, CCL5, CCL5 and CXCL2 that are essential for recruitment of leukocytes to site of inflammation were also increased (**Figure 17B**). The bacterial burden was significantly increased after

blocking trans signalling (**Figure 17C**) demonstrating the crucial role of IL-6 trans-signalling for the defense against this bacterial infection.

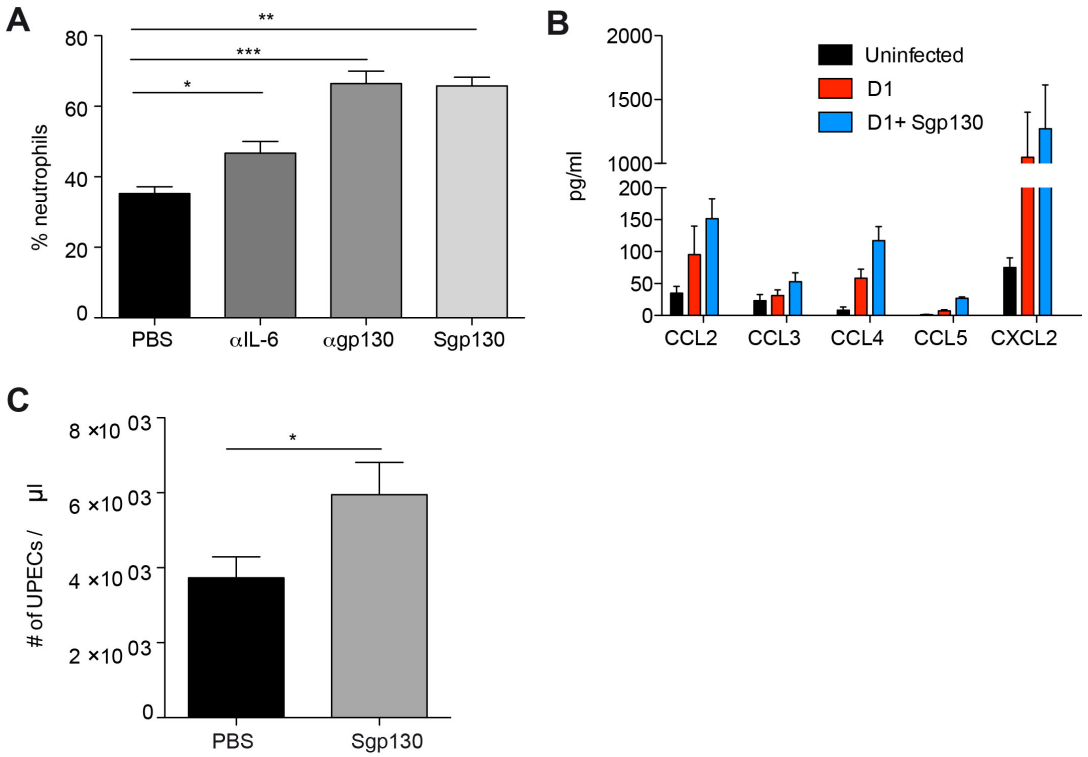

Figure 17: Blocking IL-6 trans-signalling aggravates the infection.

C57BL/6 mice were infected with UPECs and analyzed one day after infection. (A) Three hours after infection, PBS, aIL-6, agp130 or Sgp130 was instilled transurethrally to block IL-6 signalling and the frequency of neutrophils was determined by flow cytometry. (B) Bladder homogenates of infected mice was analyzed by Luminex for the expression of CCL2, CCL3, CCL4, CCL5 and CXCL2. (C) The number of bacteria was determined in the bladder one day after infection and after blocking IL-6 trans-signalling. Data are mean ± SEM, n=8.

3.1.14 Blocking IL-6 trans-signalling aggravates the infection within the epithelium and the lumen.

To further image the IL-6-dependent aggravation of the infection, C57BL/6 mice were locally injected with the anti-IL-6 antibody and infected with GFP expressing UPECs. Bladder sections were stained for DAPI (nucleus), F4/80 (macrophages), Ly6G (neutrophils) and GFP-expressing UPECs. One day after infection, numerous GFP expressing UPECs could be found in the lumen and the epithelium of bladders after anti-IL-6-treatment (**Figure 18A**).

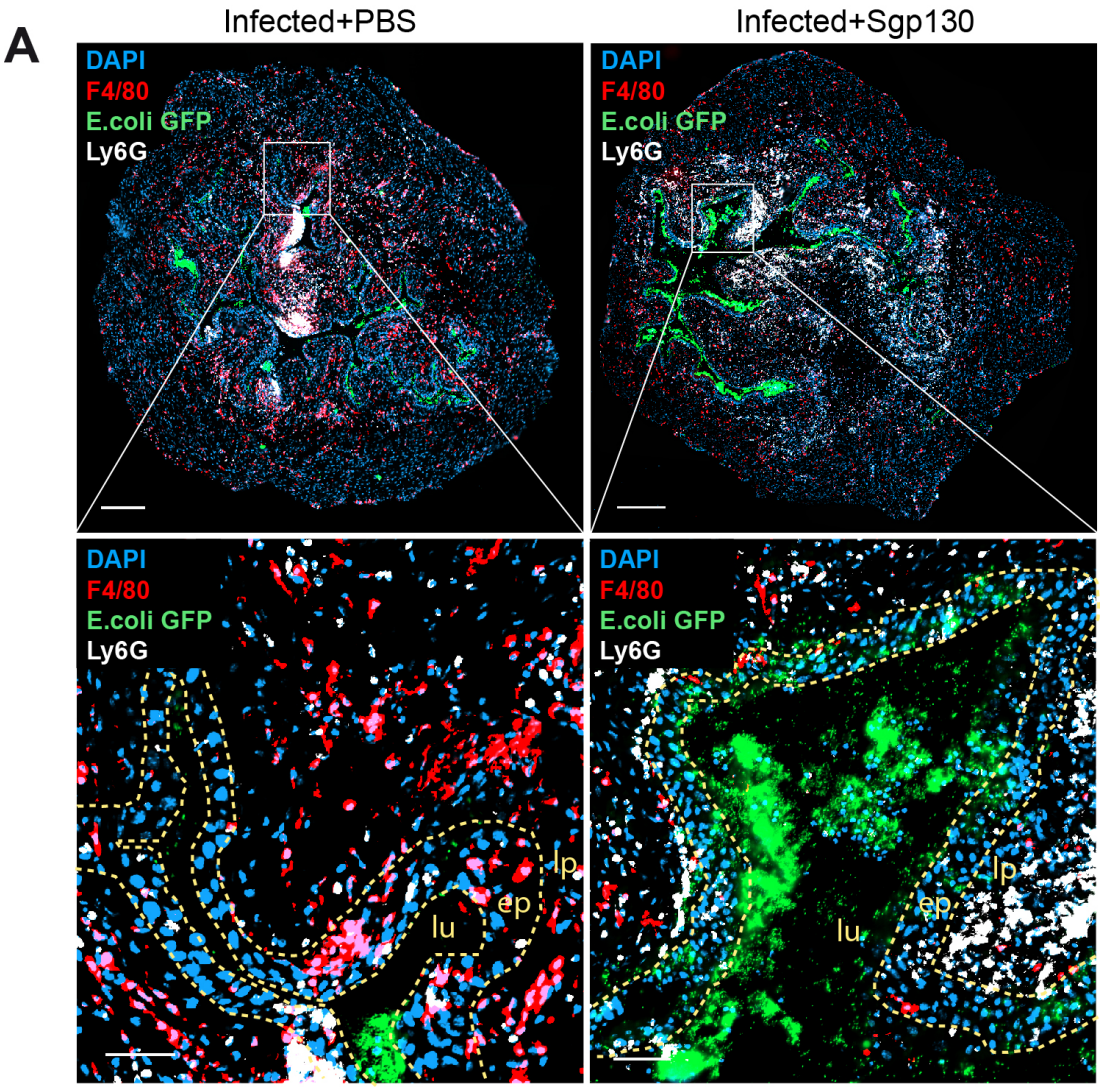

Figure 18: Blocking IL-6 trans signalling aggravated the infection within the lumen.
C57BL/6 mice were infected with GFP-UPECs and analyzed one day after infection. (A) Three hours after infection, PBS or Sgp130 was instilled transurethrally to block IL-6 signalling. The presence and localization of bacteria was determined. Data are mean ± SEM, n=5.

3.1.15 Proliferating Ly6C+ monocytes regulate the defence against the infection

The increased bacterial burden after blocking IL-6 might be due to the pleiotropic function of IL-6. To address the question whether proliferation contributes to the anti-bacterial response, the Aurora kinase inhibitor Danusertib was utilized. Aurora kinases are enzymes that are essential for cell division by regulating chromatid segregation. Aurora kinase inhibitor Danusertib inhibits all 3 kinds of Aurora kinases A, B and C. Instillation of

Danusertib directly into the bladder of the mice post infection significantly reduced the proliferation of Ly6C+ monocytes (**Figure 19A**) and blocking proliferation resulted in significant reduction in bacterial clearance as more bacterial colonies were found in Danusertib treated mice (**Figure 19B**). These data thus indicate the role of proliferating Ly6C+ monocytes in defence against UPEC infections.

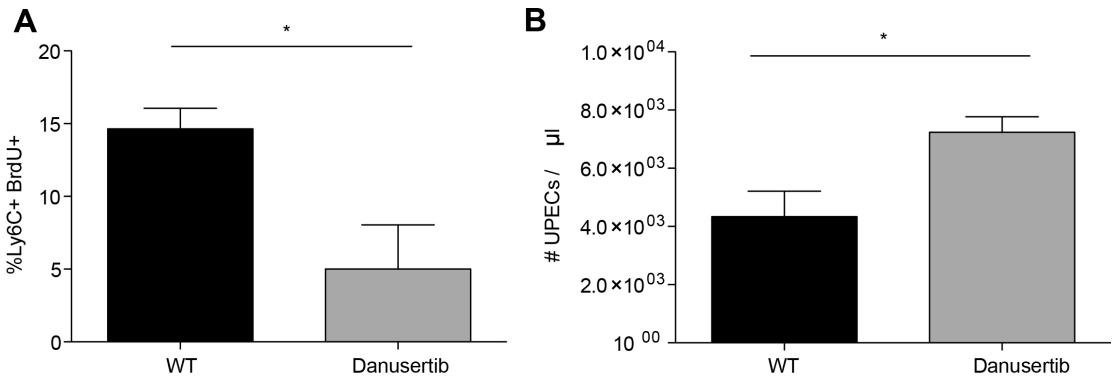

Figure 19: Proliferating Ly6C+ monocytes regulate the defence against the infection.

C57BL/6 mice were infected with UPECs and analyzed one day after infection. (A) Three hours after infection, PBS or Danusertib was instilled transurethrally to block proliferation and the frequency of Ly6C+BrdU+ cells was determined by flow cytometry. (B) Bladder digests were plated to obtain number of UPECs in each bladder post infection. Data are mean ± SEM, n=4.

3.2.1 Establishment of PN

One of the inevitable consequences of recurrent UTIs is PN. Therefore the role of monocytes was also investigated during UPEC induced bacterial infection of the kidneys. For this a protocol was optimized to induce PN. The number of UPECs in the overnight culture was determined to calculate number of UPECs inoculated. 5 × 10^9 UPECs were thus used in each experiment for induction of PN (**Figure 20A**). Furthermore, after induction of PN the number of UPECs in each infected kidney was determined by counting the CFUs (**Figure 20B and C**).

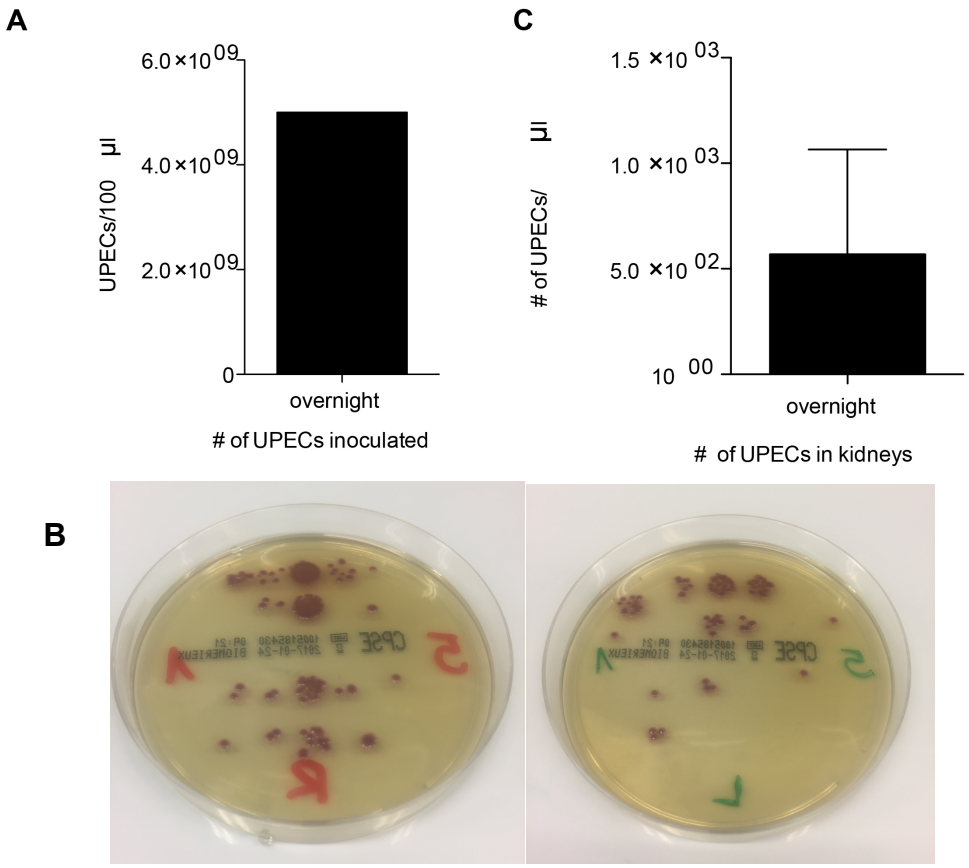

Figure 20: Establishment of PN in mice.
WT mice were transurethrally infected with UPECs twice at 3 hrs intervals to induce PN. **(A)** Number of bacterial colonies was counted in overnight-cultured UPECs. **(B)** Infected kidneys were digested and bacterial CFU were counted 24 hrs post incubation. (C) Number of CFU in the infected kidneys.

3.2.2 Accumulation of monocytes and neutrophils during PN

After PN induction, kidneys were isolated and analyzed by flow cytometry. Infection led to accumulation of neutrophils (F4/80-Gr1+) and Gr1+ inflammatory monocytes in the infected kidneys (**Figure 21A**). Further analysis was done utilizing CCR2 KO mice that lack recruitment of CCR2+ (Gr1+) monocytes into the infected tissues. In comparison to the WT mice, CCR2 KO mice had significantly reduced accumulation of Gr1+ monocytes while accumulation of neutrophils was not affected (**Figure 21A**). Further analysis revealed the accumulation of these innate immune cells over 6 days post infection. Gr1+ monocytes peak 1-day post infection and then their numbers decline whereas Gr1- macrophages showed constant accumulation over time (**Figure 21B**). Similar analysis in CCR2 KO mice revealed that this

accumulation of Gr1- macrophages over time was lacking in these mice indicating that this increase/accumulation of Gr1- macrophages might be dependent on Gr1+ monocytes. Analysis of CCR2 KO mice revealed lack of CCR2 dependent Ly6C+ cells in the infected kidneys as expected. Gr1- monocytes on the other hand show low proliferative capability as Gr1- cells in infected wild type mice.

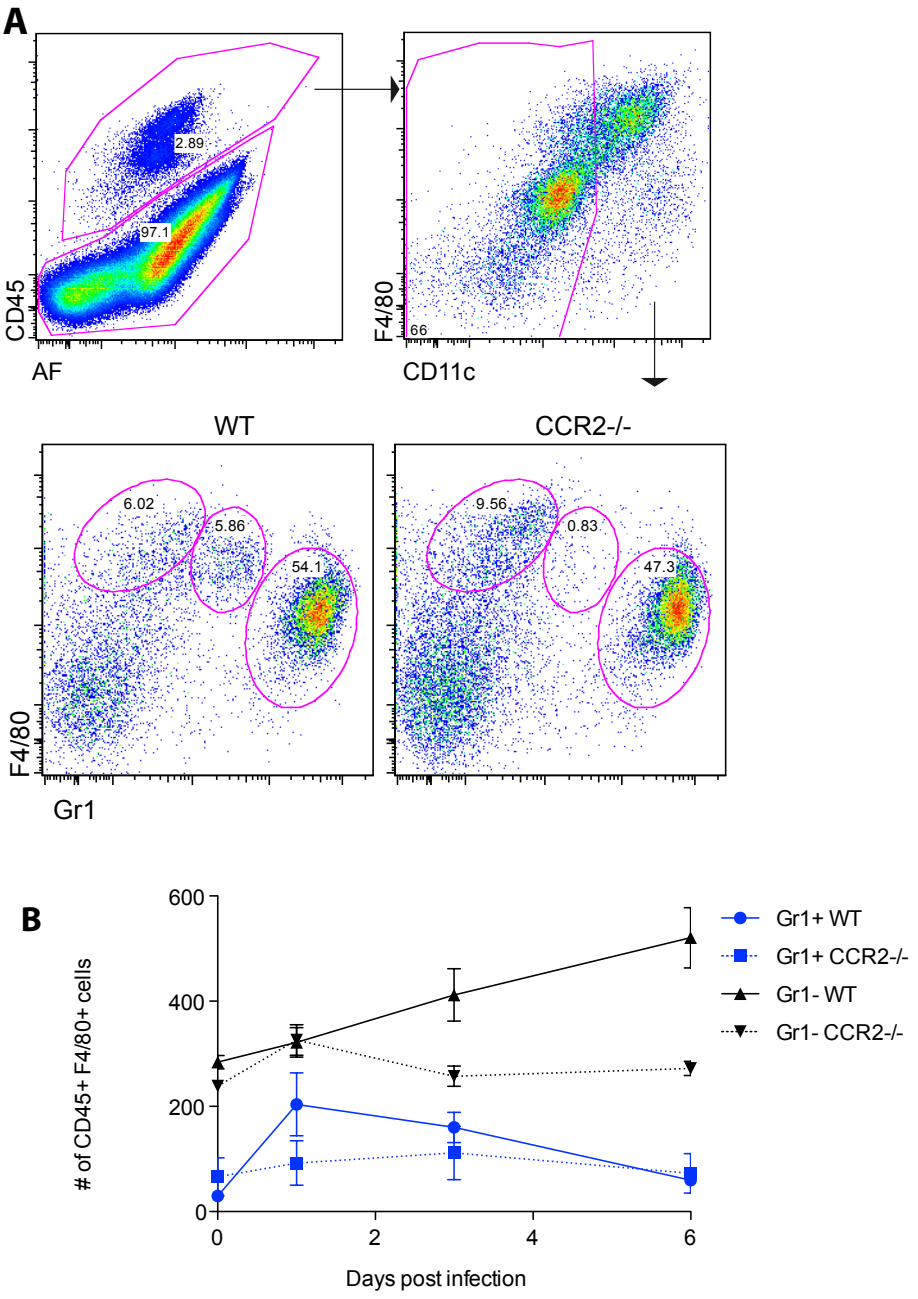

Figure 21: Kinetics of monocyte accumulation in the infected kidneys during PN.
WT and CCR2 KO mice were transurethrally infected with UPECs twice at 3 hrs intervals to induce PN. One day after infection, the kidneys were isolated and analyzed by flow cytometry. (**A**) Flow cytogram depicting the gating strategy for monocytes and neutrophils in

an infected kidney. (**B**) Frequencies of Gr1+ and Gr1- cells in the infected kidneys of WT and CCR2 KO mice over a period of 6 days. Data are mean ± SEM, n=10.

3.2.3 Absence of Ly6C+ cells leads to decreased defense against PN

To further elucidate the role of CCR2 dependent Ly6C+ (Gr1+) cells during PN, the bacterial burden in WT animals versus CCR2 KO animals was determined. In comparison to WT controls, CCR2 KO mice showed increased bacterial burden in their urine one-day post infection indicating that these cells contribute to the defence against this infection in the kidneys (**Figure 22A**). These data also indicated that there might be an important role of CCR2 dependent cells in this disease model.

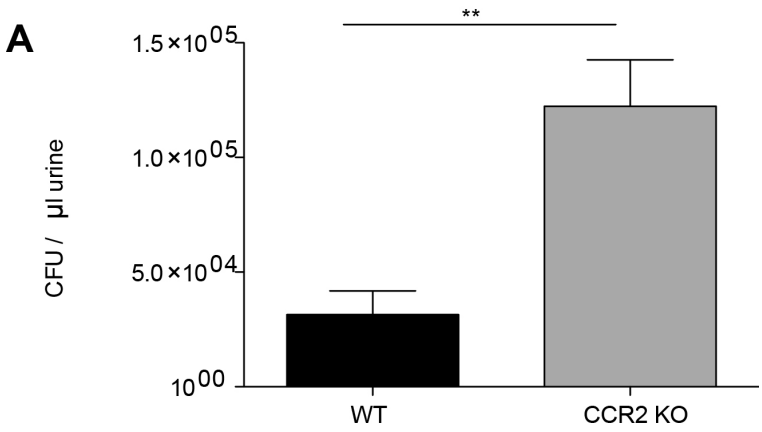

Figure 22: Absence of Ly6C+ cells leads to decreased defence against PN.
WT, CCR2 KO mice of around 12 weeks old were transurethrally infected with UPECs twice at 3 hrs intervals to induce PN. (A) Urine from these mice 1-day post infection was diluted and plated on CFU plates and the bacterial colonies were counted 1-day post incubation.

3.2.4 Local up-regulation of IL-6 in the infected organs

Next, the level of IL-6 in the blood and infected organs was measured. Level of IL-6 was significantly up regulated in the infected kidneys and the infected bladders (Figure 23B). The levels of IL-6 in blood however remained unchanged upon infection (Figure 23A). These data indicate that regulation of IL-6 dependent effects are rather local and take place in the infected organs.

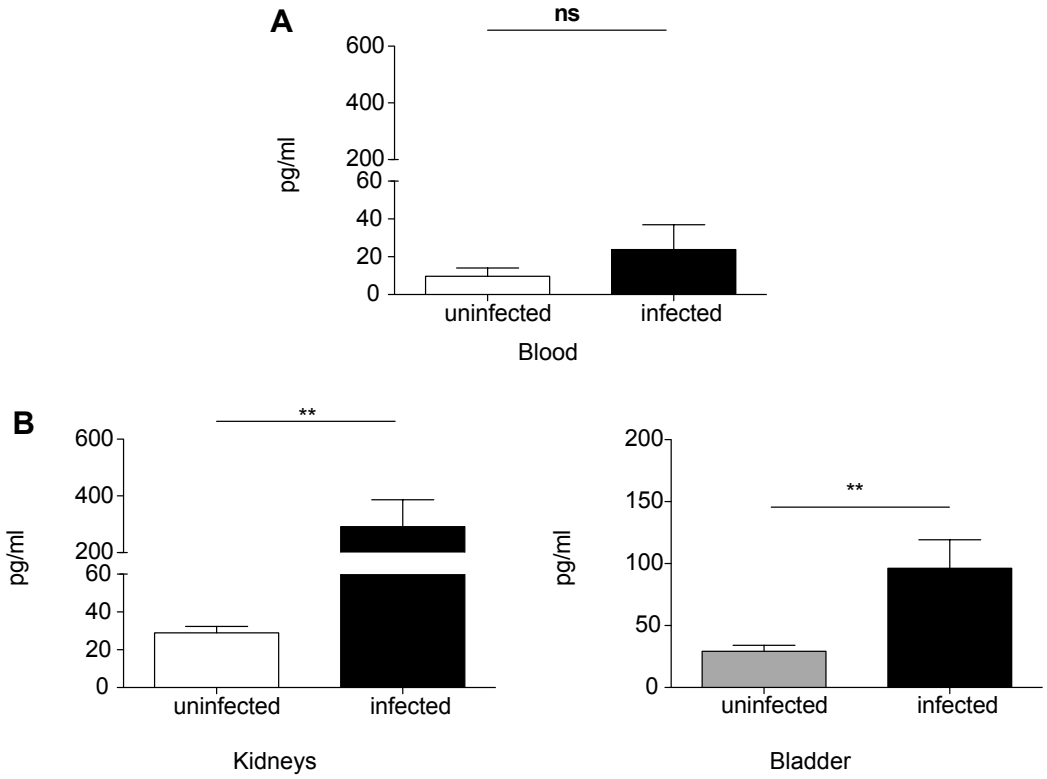

Figure 23: Local up regulation of IL-6 in the infected organs.
C57BL/6 mice were infected with UPECs and analyzed one day after infection. (**A**) Level of IL-6 was measured in blood of infected mice versus uninfected controls. (**B**) Level of IL-6 was measured in infected kidneys and bladders versus uninfected controls. Data are mean ± SEM; n=10.

3.2.5 Gr1+ monocytes proliferate in the kidneys during Pyelonephritis

Next it was investigated whether proliferation of Ly6C+ monocytes also exists in kidneys. In several cases such as during recurring infection of the bladder, ascension of UPEC from the bladder into the kidneys can take place resulting in the infection of the kidneys (Pyelonephritis). Thus, the proliferation of Ly6C+ cells was determined in the kidney during Pyelonephritis. Accumulation of Gr1+ (Ly6C+) cells was found in the kidneys post infection (**Figure 24A**). Furthermore, these cells incorporated BrdU indicating that they proliferate in the infected kidneys and a longitudinal analysis revealed accumulation of these proliferating cells in the infected kidneys (**Figure 24B**) indicating Ly6C+ monocytes also proliferate in the kidney during PN.

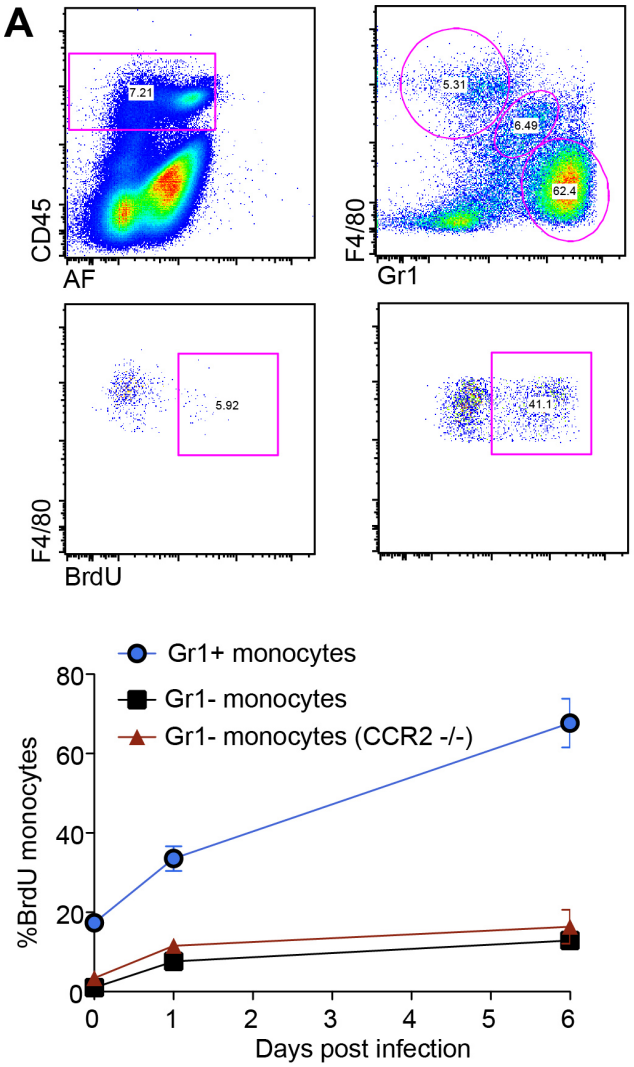

Figure 24: Gr1+ (Ly6C+) monocytes proliferate during PN.
C57BL/6 mice were transurethrally infected with UPECs. 3 hrs post infection, the mice were re-infected to allow ascension of UPEC to kidneys. (A) Accumulation of proliferating (BrdU+) Gr1 (Ly6C+) cells in the kidneys. (B) Longitudinal analysis of proliferating Gr1 (Ly6C+) cells. Data are mean ± SEM; n=10.

In conclusion, Ly6C+ monocytes proliferate during bacterial infections such as UTI and PN and also in inflamed organs. IL-6 trans-signalling regulated this proliferation as blocking experiments resulted in significantly decreased proliferation of specifically Ly6C+ monocytes in comparison to Ly6C- monocytes. These results also reveal that lack of proliferating Ly6C+ monocytes resulted in increased bacterial burden indicating their role in role in bacterial defence and non-proliferating Ly6C+ monocytes assist in phagocytosis of UPECs in the infected bladders.

4. Discussion

Uropathogenic *Escherichia coli* (UPEC) cause the majority of urinary tract infections. Since the bladder compartment is constantly in contact with urine and other toxic components in urine,, it is important to have a tightly regulated immune response. Bladder epithelial cells are an essential component of the immune response as they produce chemokines, cytokines and other anti-bacterial components to recruit and activate immune cells (Mulvey et al., 2001).. Neutrophils are the first immune cells recruited to the site of infection to clear bacteria in UTI (Moore et al., 1996; Sakakibara and Tosato, 2011). They are found in the urine of mice as early as 2 hrs post infection and peak at around 6 hours (Fielding et al., 2005). Macrophages on the other hand are abundant as a resident population in the bladder. Monocytes are also recruited from circulation after infection. The coordination of resident macrophages and recruited monocytes has been shown recently to be an essential component of the immune responses against UTI. This study showed that coordination of these cell types allowed migration of neutrophils deep into epithelium in a MMP9 dependent manner where they can perform their effector functions (Schiwon et al., 2014). Furthermore, absence of recruited monocytes in CCR2 -/- mice results in impaired bacterial clearance (Scheller et al., 2011).

Ascending infections with UPECs lead to pyelonephritis, which is one of the most prevalent kidney infections. Infections of the kidneys are rather focal resulting in scarring of surrounding tissue and eventually leading to kidney fibrosis and loss of renal function. While the pathology of bladder infections is well described, pathology of PN is poorly understood. DCs are an abundant cell type in the kidney and their role in renal diseases is well studied. Kidney DCs express CD11c+ and MHCII+ (Mroczko et al., 2010; Panichi et al., 2004) and these cells have been shown to play a sentinel role in PN (Bruttger et al., 2015; Hashimoto et al., 2013; Jenkins et al., 2011). TLR expressing kidney DCs have also been shown to detect UPECs and contribute to the defense against bacterial PN by production of chemokines that attract neutrophils

(Jostock et al., 2001). The role of macrophages in kidney diseases has also been investigated. Macrophages were shown to be recruited during renal glomerulonephritis (Shi et al., 2011) and produce cytokines like IL-1 and TNFa (Haraoka et al., 1999) suggesting their role in pathogenesis of renal diseases. In a model of unilateral ureteral obstruction (UUO), depletion of macrophages in the early phase of inflammation increased renal fibrosis. On the other hand, depletion of macrophage during resolution phase reduced fibrosis (Agace et al., 1995). Macrophages were also shown to exacerbate renal injury in a model of human glomerulosclerosis (Engel et al., 2008) and a protective function of macrophages were described in a model of chronic kidney disease (CKD) (Kruger et al., 2004; Weisheit et al., 2015).

These studies indicate that monocytes and macrophages play an important role during the pathogenesis of renal diseases.. Whether they play a role in UTI and PN is not known. The mechanisms that regulate the abundance of monocytes and macrophages in the infected organ are still unclear. In this study the role of monocytes and macrophages during UTI and PN was investigated to extend our current understanding on their proliferative capacity during bacterial infections.

4.1 Monocytes and macrophages during UPEC infections

Over the last decades, macrophages have emerged as important protagonists in inflammatory and infectious situations (Hochheiser et al., 2011a; Hochheiser et al., 2011b; Tittel et al., 2011). The majority of these cells originate from circulating Ly6C+ monocytes, which are rapidly recruited from the blood (Hochheiser et al., 2013). Local accumulation of blood-derived monocytes has been considered crucial during infections and in chronic inflammatory diseases (Ferrario et al., 1985). The fact that circulating monocytes are efficiently recruited to the site of inflammation established the paradigm that local proliferation of these cells does not exist and cell cycle arrest cannot be overcome. However, recent studies showed the local proliferation of macrophages in different disease models. Proliferation of Ly6C+ macrophages during atherosclerosis in arterial lesions indicated cell

cycle exit of monocyte-derived macrophages (Matsumoto, 1990; Tipping et al., 1991). There is also further evidence of local proliferation of tissue resident macrophages in a Th2-mediated disease model of helminth infections in the peritoneum (Wang and Harris, 2011). This thesis provides direct evidence that recruited Ly6C+ monocytes rapidly start to proliferate in an infected bladder. Furthermore, this proliferation takes place in an IL-6-dependent manner during acute bacterial infections and this proliferation is crucial for defense against bacterial urinary tract infection. Analysis of the localization of immune cells within the bladder using immunohistochemistry revealed that proliferating monocytes were located to the lamina propria. Endothelial cells are a major cell type that is present in the lamina propria. The have been shown to be a major source for IL-6 after stimulation with proinflammatory mediators, such as IL-1β or TNFα (Wang et al., 2008). It was found that during urinary tract infection, non-hematopoietic cells mainly produced the proinflammatory molecule IL-6 suggesting that invading monocytes are stimulated by IL-6 produced by endothelial cells to proliferate after tissue entry. As opposed to proliferating monocytes, non-proliferating monocytes were localized in the infected uroepithelium, in close proximity to neutrophils. Previously, macrophages have been shown to detect „eat-me“ signals on neutrophils to phagocytose matured neutrophils (Lu et al., 2013). This process was termed efferocytosis, which has long been appreciated for its role in the resolution of inflammation and restoration of tissue homeostasis. In this murine model of urinary tract infection, uptake of apoptotic neutrophils was facilitated by non-proliferating Ly6C+ monocytes identifying a novel role for this cell type during urinary tract infection. However, the impact of this process needs to be defined.

Proliferation of Ly6C+ monocytes also occurs in other organs than the bladder. Ly6C+ proliferating monocytes were found in the infected kidneys during pyelonephritis and in the inflamed peritoneum in the LPS-induced model of peritonitis. These data suggest that proliferation of Ly6C+ monocytes exists in an organ independent manner and might play a crucial rule during a wide range of inflammation and infectious settings. These data

also makes it of great relevance to understand the mechanisms that regulate the local proliferation of recruited Ly6C+ monocytes for targeting proliferation and thus accumulation of monocytes in chronic inflammatory disease models.

Ly6C- tissue resident macrophages on the other hand are known to promote the formation of extracellular matrix and thus contribute in wound healing (Murray and Wynn, 2011; Shi and Pamer, 2011). Using CCR2 KO mice that lack recruitment of Ly6C+ monocytes, reduced accumulation of Ly6C- monocytes was seen indicating that Ly6C+ inflammatory monocytes are essential component also of the resolution phase of the infection. Lack of Ly6C+ inflammatory monocytes in CCR2 KO mice, not only resulted in reduced accumulation of resident Ly6C- macrophages over time but as well as increased bacterial burden. Differentiation capacity of Ly6C+ to Ly6C- macrophage under homeostasis and as well as in inflammation has been well described (Gordon and Mantovani, 2011; Gordon and Taylor, 2005; Murray et al., 2014). The data in this study also suggest that differentiation of Ly6C+ monocytes to a resident phenotype might occur which fails to happen in CCR2 KO mice indicating plasticity of this cell subset. These differentiated macrophages then along with other tissue resident macrophages might contribute to the wound healing process. These data thus indicate that Ly6C+ monocytes proliferate to increase in numbers and then differentiate to resident cell type and contribute to the resolution of the renal infection. This step can be thus a major step in the pathology of renal infections such as PN. However, how this differentiation is regulated and to find the impact of the lack of these cells on disease outcome such as on renal fibrosis will be an interesting target for the future to improve our understanding on the pathology of PN.

4.2 Role of IL-6 signaling in defense against UTI

Over the last decade, rapid progress has been achieved to better understand the pleiotropic function of the potent proinflammatory molecule IL-6 (Motwani and Gilroy, 2015; Robbins et al., 2013). Recent studies reveal that IL-6 trans-signaling induces the proinflammatory functionality of leukocytes (Robbins et

al., 2013), whereas anti-inflammatory properties of IL-6 were mediated by classical signaling (Robbins et al., 2013). Major advances have been achieved by blocking IL-6 in a variety of inflammatory settings. In arthritis, blocking IL-6 prevents the perpetuation of the disease (Davies et al., 2013b; Jenkins et al., 2011) and most importantly, the accumulation of monocytes (Scheller et al., 2011). In contrast, overexpression of IL-6 signaling during human herpes virus 8 infections promotes the accumulation of monocytes and the development of Kaposi´s sarcoma (Savill and Fadok, 2000) indicating IL-6 as an important molecule for monocyte accumulation in a variety of inflammatory settings.

In this study, blocking IL-6 trans-signaling by topical administration of anti IL-6 or gp130 resulted in significantly reduced proliferation of Ly6C+ monocytes and the defense against the infection. Blocking of IL-6 or gp130 signaling however does not differentiate between IL-6 classical or trans-signaling. To further elucidate which type of IL-6 signaling regulates this proliferation, we blocked trans-signaling by administration of the soluble form of gp130 (Sgp130). Sgp130 is the natural inhibitor of IL-6 trans signaling. During homeostasis, it binds to the IL-6 / sIL-6R complex thereby preventing the binding of the complex to the membrane bound gp130. This step is essential for inducing IL-6 trans signaling. Injecting mice with recombinant Sgp130 thereby prevents induction of IL-6 trans signaling. Blocking Sgp130 significantly reduced monocyte proliferation and the defense against the infection. An important component of the IL-6 trans-signaling is the shed IL-6 receptor. Myeloid cells, such as monocyte and neutrophils have been considered as a crucial source for this shed receptor (Rogers et al., 2014) and inflammatory mediators released by neutrophils induce the shedding of the IL-6 receptor in an ADAM17-dependent fashion (Yang et al., 2014; Yona et al., 2013). However, proliferation of Ly6C+ monocytes was unchanged in *LysM* ^{cre} *x IL6r* $^{flox/flox}$ animals indicating that shedding the IL-6 receptor by other cells than monocytes and neutrophils facilitate IL-6 trans-signaling in this infection model. These data suggest that IL-6 does not act directly on Lysozyme-positive cells such as monocytes and neutrophils. Thus, classical IL-6

signaling in monocytes is dispensable for their proliferation.

These data show that IL-6 does not act directly on monocytes and neutrophils indicating involvement of a mediator molecule. IL-6 has also already been known to be involved in modulating CD4 T cell responses by acting as a mediator (Hunter and Jones, 2015). It has been shown to induce IL-4 production from activated CD4 T cells that further helps in Th2 differentiation (Rose-John, 2012). IL-6 has also been shown to promote Th17 differentiation via TGF-ß (Rose-John, 2012). This knowledge suggests that IL-6 could act on a mediator molecule also in this model, where it further promotes proliferation of Ly6C+ monocytes. It would be interesting to identify this mediator molecule to better understand the mechanism of IL-6 induced proliferation.

4.3 Role of proliferating monocytes in defense against UTI.

Ly6C+ inflammatory monocytes not only proliferated in the infected bladder but also vigorously proliferate in the infected kidneys suggesting importance of these cells in the defense against UPEC and associated infections such as cystitis and pyelonephritis. The role of Ly6C+ monocytes in the defense against infections has already been studied in CCR2 KO mice where lack of these cells result in increased bacterial burden (Scheller et al., 2011). This study revealed that these proliferating Ly6C+ monocytes contributed to defense against this infection as specific lack of these proliferating cells resulted in increased bacterial burden (Figure 25). Specific blocking was achieved by the aurora kinase inhibitor Danusertib. Of note, danusertib can inhibit proliferation of many different cell types that exists in the bladder. A cell specific blocking of Ly6C+ monocytes is not possible with the current techniques available. The development of novel techniques is required to more specifically block the proliferation of Ly6C+ monocytes.

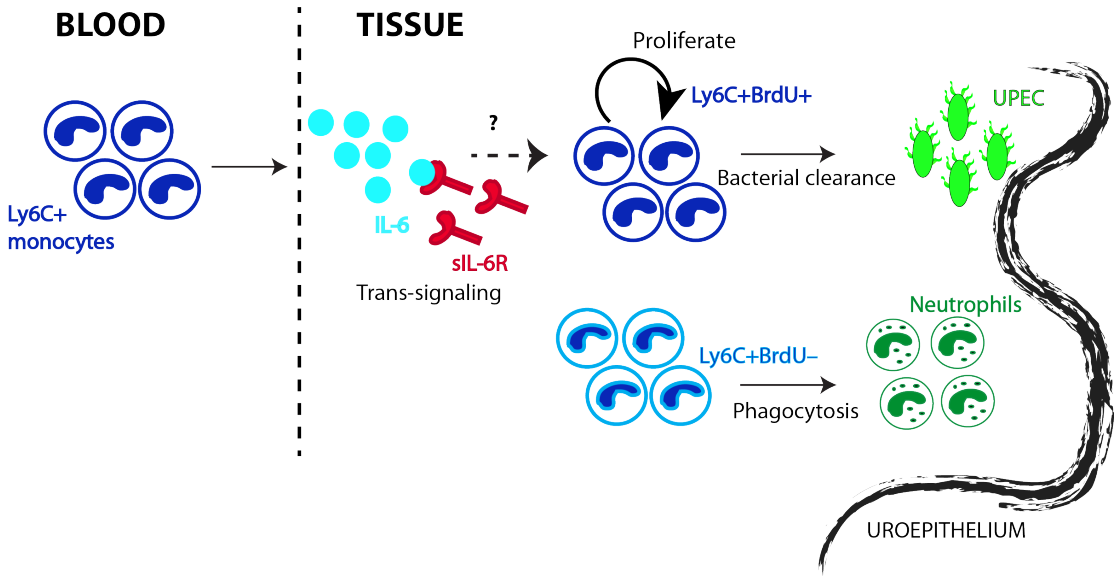

Figure 25: Schematic summary of IL-6 induced proliferation of Ly6C+ monocytes.
Ly6C+ monocytes that enter the tissue from blood stream proliferate in an IL-6 trans-signalling dependent manner. These proliferating Ly6C+ monocytes then contribute to bacterial clearance while Ly6C+ non-proliferating monocytes perform phagocytosis.

An interesting finding was increased numbers of neutrophils when proliferation of Ly6C+ monocytes was blocked using Sgp130. Neutrophils play an essential role in defense against UTI by migrating into the epithelium where they perform the effector functions (Gordon and Taylor, 2005). Localization of neutrophils in this model of UTI also provides hints about their functionality. It indicates a possible coordination of Ly6C+ proliferating monocytes and neutrophils. Crosstalk between neutrophils and monocytes has already been well described (Schiwon et al., 2014). Neutrophils are known to perform various functions such as phagocytosis, release of reactive oxygen species (ROS) and formation of neutrophil extracellular traps (NETs) (Brinkmann et al., 2004; Kaplan and Radic, 2012). Analysis of neutrophil functionality within uroepithelium would provide us functional capabilities of neutrophils that reach the uroepithelium. Whether neutrophil that reaches uroepithelium in the absence of proliferating monocytes, is still capable of efficiently forming NETs or doing phagocytosis.

These data extend our current knowledge on the mechanism that regulate monocyte accumulation during inflammation and infections. This thesis

highlights the importance of local proliferation of monocytes, which was for long not appreciated. Furthermore, it gives us hints about the functionality of Ly6C+ monocytes in this particular disease model. Proliferating Ly6C+ monocytes contribute to defence against the infection while non-proliferating monocytes perform phagocytosis. This thesis reveals a novel function of IL-6 and extends the current knowledge on the pleiotropic molecule IL-6.

5. Conclusion and Outlook

In summary, this study demonstrates the local proliferation of recruited Ly6C+ monocytes in infected tissues. This novel finding questions the current dogma that monocytes in peripheral tissues are unable to overcome cell cycle arrest. The capability of monocytes to overcome cell cycle arrest and proliferate might also explain the recent findings that macrophages are able to proliferate in an inflammatory microenvironment. This study shows that Ly6C+ monocytes actively proliferate in infected and inflamed organs. IL-6 trans-signaling was identified as the regulator for local monocyte proliferation. Furthermore, this effect was specific for Ly6C+ monocytes as Ly6C- macrophages showed barely any proliferation. The proliferation of these cells in the infected bladder and the kidney, as well as in the inflamed peritoneum suggests that proliferation is an organ-overarching mechanism.

This study also suggests that proliferating Ly6C+ monocytes are crucial to prevent UTI. However, the lack of cell-specific blocking techniques demands for further experiments to specifically elucidate the role of these in UTI. Animals such as CycB1-GFP mice, which express the enhanced green fluorescent protein (EGFP) under the Cyclin B1 (*Ccnb1*) promoter might help to elucidate the functionality of these cells (Klochendler et al., 2012). Proliferating monocytes should be sorted from infected animals to perform genomic and proteomic analysis. MALDI-Imaging, which analyses the cellular proteome on histological sections, would also provide novel insights on the role of these cells during infections. These techniques might also be applicable to analyze the role of non-proliferative Ly6C+ monocytes. This study provides direct evidence, that these cells performed phagocytosis of neutrophil, which might contribute to clearance of inflammation. However, further experiments are required to delineate the functionality of these cells and to functionally differentiate these cells from proliferating monocytes. Revealing functional information on proliferating versus no proliferating monocytes would help us better understand disease pathogenesis.

This study shows that IL-6 regulates the proliferation of Ly6C+ monocytes. However, it remained unclear whether IL-6 acts directly on monocytes to induce proliferation. Bone marrow chimeric mice, which received a mixture of cells from IL6R KO and CCR2 KO mice, might help addressing this question. Further, adoptive transfer of cells that have been blocked *ex vivo* with proliferation inhibitors might offer another alternative in determining if these cells respond to IL-6 to proliferate.

Finally, this study might explain why IL-6 plays such a crucial role in regulating immunity in many inflammatory disorders, such as arthritis, inflammatory bowels disease etc. It also highlights the beneficial role of targeting IL-6 to prevent monocyte accumulation, inflammation and pathogenesis. Further mechanistic insights into how IL-6 trans-signalling regulates bacterial clearance will help in developing specific targeting strategies against chronic inflammatory diseases such as rheumatoid arthritis. Lastly, this knowledge advances our understanding on the pathogenesis during bacterial infections and inflammation.

References:

Agace, W.W., Hedges, S.R., Ceska, M., and Svanborg, C. (1993). Interleukin-8 and the neutrophil response to mucosal gram-negative infection. J clin invest *92*, 780-785.

Agace, W.W., Patarroyo, M., Svensson, M., Carlemalm, E., and Svanborg, C. (1995). Escherichia coli induces transuroepithelial neutrophil migration by an intercellular adhesion molecule-1-dependent mechanism. Infect Immun *63*, 4054-4062.

Ajami, B., Bennett, J.L., Krieger, C., Tetzlaff, W., and Rossi, F.M. (2007). Local self-renewal can sustain CNS microglia maintenance and function throughout adult life. Nature neuroscience *10*, 1538-1543.

Ajuebor, M.N., Das, A.M., Virag, L., Flower, R.J., Szabo, C., and Perretti, M. (1999). Role of resident peritoneal macrophages and mast cells in chemokine production and neutrophil migration in acute inflammation: evidence for an inhibitory loop involving endogenous IL-10. Journal of immunology *162*, 1685-1691.

Auffray, C., Fogg, D., Garfa, M., Elain, G., Join-Lambert, O., Kayal, S., Sarnacki, S., Cumano, A., Lauvau, G., and Geissmann, F. (2007). Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. Science *317*, 666-670.

Bataille, R., Robillard, N., Pellat-Deceunynck, C., and Amiot, M. (2003). A cellular model for myeloma cell growth and maturation based on an intraclonal CD45 hierarchy. Immunol Rev *194*, 105-111.

Bethin, K.E., Vogt, S.K., and Muglia, L.J. (2000). Interleukin-6 is an essential, corticotropin-releasing hormone-independent stimulator of the adrenal axis during immune system activation. Proc Natl Acad Sci U S A *97*, 9317-9322.

Bishop, B.L., Duncan, M.J., Song, J., Li, G., Zaas, D., and Abraham, S.N. (2007). Cyclic AMP-regulated exocytosis of Escherichia coli from infected bladder epithelial cells. Nat Med *13*, 625-630.

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D.S., Weinrauch, Y., and Zychlinsky, A. (2004). Neutrophil extracellular traps kill bacteria. Science *303*, 1532-1535.

Bruttger, J., Karram, K., Wortge, S., Regen, T., Marini, F., Hoppmann, N., Klein, M., Blank, T., Yona, S., Wolf, Y.*, et al.* (2015). Genetic Cell Ablation Reveals Clusters of Local Self-Renewing Microglia in the Mammalian Central Nervous System. Immunity *43*, 92-106.

Cailhier, J.F., Sawatzky, D.A., Kipari, T., Houlberg, K., Walbaum, D., Watson, S., Lang, R.A., Clay, S., Kluth, D., Savill, J.*, et al.* (2006). Resident pleural macrophages are key orchestrators of neutrophil recruitment in pleural inflammation. Am J Respir Crit Care Med *173*, 540-547.

Chalaris, A., Garbers, C., Rabe, B., Rose-John, S., and Scheller, J. (2011). The soluble Interleukin 6 receptor: generation and role in inflammation and cancer. European journal of cell biology *90*, 484-494.

Chorro, L., Sarde, A., Li, M., Woollard, K.J., Chambon, P., Malissen, B., Kissenpfennig, A., Barbaroux, J.B., Groves, R., and Geissmann, F. (2009). Langerhans cell (LC) proliferation mediates neonatal development, homeostasis, and inflammation-associated expansion of the epidermal LC network. The Journal of experimental medicine *206*, 3089-3100.

Chromek, M., Tullus, K., Hertting, O., Jaremko, G., Khalil, A., Li, Y.H., and Brauner, A. (2003). Matrix metalloproteinase-9 and tissue inhibitor of metalloproteinases-1 in acute pyelonephritis and renal scarring. Pediatric research *53*, 698-705.

Colomiere, M., Ward, A.C., Riley, C., Trenerry, M.K., Cameron-Smith, D., Findlay, J., Ackland, L., and Ahmed, N. (2009). Cross talk of signals between EGFR and IL-6R through JAK2/STAT3 mediate epithelial-mesenchymal transition in ovarian carcinomas. British journal of cancer *100*, 134-144.

Dann, S.M., Spehlmann, M.E., Hammond, D.C., Iimura, M., Hase, K., Choi, L.J., Hanson, E., and Eckmann, L. (2008). IL-6-dependent mucosal protection prevents establishment of a microbial niche for attaching/effacing lesion-forming enteric bacterial pathogens. Journal of immunology *180*, 6816-6826.

Davies, L.C., Jenkins, S.J., Allen, J.E., and Taylor, P.R. (2013a). Tissue-resident macrophages. Nat Immunol *14*, 986-995.

Davies, L.C., Rosas, M., Jenkins, S.J., Liao, C.T., Scurr, M.J., Brombacher, F., Fraser, D.J., Allen, J.E., Jones, S.A., and Taylor, P.R. (2013b). Distinct bone marrow-derived and tissue-resident macrophage lineages proliferate at key stages during inflammation. Nature communications *4*, 1886.

Davies, L.C., Rosas, M., Smith, P.J., Fraser, D.J., Jones, S.A., and Taylor, P.R. (2011). A quantifiable proliferative burst of tissue macrophages restores homeostatic macrophage populations after acute inflammation. Eur J Immunol *41*, 2155-2164.

Diao, H., and Kohanawa, M. (2005). Endogenous interleukin-6 plays a crucial protective role in streptococcal toxic shock syndrome via suppression of tumor necrosis factor alpha production. Infect Immun *73*, 3745-3748.

Dube, P.H., Handley, S.A., Lewis, J., and Miller, V.L. (2004). Protective role of interleukin-6 during Yersinia enterocolitica infection is mediated through the modulation of inflammatory cytokines. Infect Immun *72*, 3561-3570.

Duffield, J.S., Tipping, P.G., Kipari, T., Cailhier, J.F., Clay, S., Lang, R., Bonventre, J.V., and Hughes, J. (2005). Conditional ablation of macrophages halts progression of crescentic glomerulonephritis. Am J Pathol *167*, 1207-1219.

Engel, D.R., Maurer, J., Tittel, A.P., Weisheit, C., Cavlar, T., Schumak, B., Limmer, A., van Rooijen, N., Trautwein, C., Tacke, F.*, et al.* (2008). CCR2 mediates homeostatic and inflammatory release of Gr1(high) monocytes from the bone marrow, but is dispensable for bladder infiltration in bacterial urinary tract infection. Journal of immunology *181*, 5579-5586.

Epelman, S., Lavine, K.J., and Randolph, G.J. (2014). Origin and functions of tissue macrophages. Immunity *41*, 21-35.

Eto, D.S., Jones, T.A., Sundsbak, J.L., and Mulvey, M.A. (2007). Integrin-mediated host cell invasion by type 1-piliated uropathogenic Escherichia coli. PLoS Pathog *3*, e100.

Ferrario, F., Castiglione, A., Colasanti, G., Barbiano di Belgioioso, G., Bertoli, S., and D'Amico, G. (1985). The detection of monocytes in human glomerulonephritis. Kidney Int *28*, 513-519.

Fielding, C.A., McLoughlin, R.M., Colmont, C.S., Kovaleva, M., Harris, D.A., Rose-John, S., Topley, N., and Jones, S.A. (2005). Viral IL-6 blocks neutrophil infiltration during acute inflammation. Journal of immunology *175*, 4024-4029.

Fischer, H., Yamamoto, M., Akira, S., Beutler, B., and Svanborg, C. (2006). Mechanism of pathogen-specific TLR4 activation in the mucosa: fimbriae, recognition receptors and adaptor protein selection. Eur J Immunol *36*, 267-277.

Flores-Mireles, A.L., Walker, J.N., Caparon, M., and Hultgren, S.J. (2015). Urinary tract infections: epidemiology, mechanisms of infection and treatment options. Nat Rev Microbiol.

Foxman, B. (2002). Epidemiology of urinary tract infections: incidence, morbidity, and economic costs. The American journal of medicine *113 Suppl 1A*, 5S-13S.

Geissmann, F., Gordon, S., Hume, D.A., Mowat, A.M., and Randolph, G.J. (2010). Unravelling mononuclear phagocyte heterogeneity. Nature rev immunol *10*, 453-460.

Geissmann, F., Jung, S., and Littman, D.R. (2003). Blood monocytes consist of two principal subsets with distinct migratory properties. Immunity *19*, 71-82.

Ginhoux, F., and Jung, S. (2014). Monocytes and macrophages: developmental pathways and tissue homeostasis. Nat Rev Immunol *14*, 392-404.

Godaly, G., Proudfoot, A.E., Offord, R.E., Svanborg, C., and Agace, W.W. (1997). Role of epithelial interleukin-8 (IL-8) and neutrophil IL-8 receptor A in Escherichia coli-induced transuroepithelial neutrophil migration. Infect Immun *65*, 3451-3456.

Gordon, S., and Mantovani, A. (2011). Diversity and plasticity of mononuclear phagocytes. Eur J Immunol *41*, 2470-2472.

Gordon, S., and Taylor, P.R. (2005). Monocyte and macrophage heterogeneity. Nat Rev Immunol *5*, 953-964.

Guiteras, R., Flaquer, M., and Cruzado, J.M. (2016). Macrophage in chronic kidney disease. Clinical kidney journal *9*, 765-771.

Haraoka, M., Hang, L., Frendeus, B., Godaly, G., Burdick, M., Strieter, R., and Svanborg, C. (1999). Neutrophil recruitment and resistance to urinary tract infection. J Infect Dis *180*, 1220-1229.

Hashimoto, D., Chow, A., Noizat, C., Teo, P., Beasley, M.B., Leboeuf, M., Becker, C.D., See, P., Price, J., Lucas, D*., et al.* (2013). Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes. Immunity *38*, 792-804.

Heinrich, P.C., Behrmann, I., Haan, S., Hermanns, H.M., Muller-Newen, G., and Schaper, F. (2003). Principles of interleukin (IL)-6-type cytokine signalling and its regulation. Biochem J *374*, 1-20.

Hochheiser, K., Engel, D.R., Hammerich, L., Heymann, F., Knolle, P.A., Panzer, U., and Kurts, C. (2011a). Kidney Dendritic Cells Become Pathogenic during Crescentic Glomerulonephritis with Proteinuria. J Am Soc Nephrol *22*, 306-316.

Hochheiser, K., Heuser, C., Krause, T.A., Teteris, S., Ilias, A., Weisheit, C., Hoss, F., Tittel, A.P., Knolle, P.A., Panzer, U*., et al.* (2013). Exclusive CX3CR1 dependence of kidney DCs impacts glomerulonephritis progression. J Clin Invest *123*, 4242-4254.

Hochheiser, K., Tittel, A., and Kurts, C. (2011b). Kidney dendritic cells in acute and chronic renal disease. International journal of experimental pathology *92*, 193-201.

Hooton, T.M., and Stam, W.E. (1991). Management of acute uncomplicated urinary tract infection in adults. The Medical clinics of North America *75*, 339-357.

Hooton, T.M., and Stamm, W.E. (1997). Diagnosis and treatment of uncomplicated urinary tract infection. Infect Dis Clin North Am *11*, 551-581.

Hunter, C.A., and Jones, S.A. (2015). IL-6 as a keystone cytokine in health and disease. Nat Immunol *16*, 448-457.

Hurst, S.M., McLoughlin, R.M., Monslow, J., Owens, S., Morgan, L., Fuller, G.M., Topley, N., and Jones, S.A. (2002). Secretion of oncostatin M by infiltrating neutrophils: regulation of IL-6 and chemokine expression in human mesothelial cells. Journal of immunology *169*, 5244-5251.

Imig, J.D., and Ryan, M.J. (2013). Immune and inflammatory role in renal disease. Comprehensive Physiology *3*, 957-976.

Jenkins, S.J., Ruckerl, D., Cook, P.C., Jones, L.H., Finkelman, F.D., van Rooijen, N., MacDonald, A.S., and Allen, J.E. (2011). Local macrophage proliferation, rather than recruitment from the blood, is a signature of TH2 inflammation. Science *332*, 1284-1288.

Jenkins, S.J., Ruckerl, D., Thomas, G.D., Hewitson, J.P., Duncan, S., Brombacher, F., Maizels, R.M., Hume, D.A., and Allen, J.E. (2013). IL-4 directly signals tissue-resident macrophages to proliferate beyond homeostatic levels controlled by CSF-1. The Journal of experimental medicine *210*, 2477-2491.

Jones, C.H., Pinkner, J.S., Roth, R., Heuser, J., Nicholes, A.V., Abraham, S.N., and Hultgren, S.J. (1995). FimH adhesin of type 1 pili is assembled into a fibrillar tip structure in the Enterobacteriaceae. Proc Natl Acad Sci U S A *92*, 2081-2085.

Jones, S.A. (2005). Directing transition from innate to acquired immunity: defining a role for IL-6. Journal of immunology *175*, 3463-3468.

Jones, S.A., Novick, D., Horiuchi, S., Yamamoto, N., Szalai, A.J., and Fuller, G.M. (1999). C-reactive protein: a physiological activator of interleukin 6 receptor shedding. The Journal of experimental medicine *189*, 599-604.

Jostock, T., Mullberg, J., Ozbek, S., Atreya, R., Blinn, G., Voltz, N., Fischer, M., Neurath, M.F., and Rose-John, S. (2001). Soluble gp130 is the natural inhibitor of soluble interleukin-6 receptor transsignaling responses. Eur J Biochem *268*, 160-167.

Justice, S.S., Hunstad, D.A., Seed, P.C., and Hultgren, S.J. (2006). Filamentation by Escherichia coli subverts innate defenses during urinary tract infection. Proc Natl Acad Sci U S A *103*, 19884-19889.

Kaplan, M.J., and Radic, M. (2012). Neutrophil extracellular traps: double-edged swords of innate immunity. Journal of immunology *189*, 2689-2695.

Kawai, T., and Akira, S. (2006a). Innate immune recognition of viral infection. Nat Immunol *7*, 131-137.

Kawai, T., and Akira, S. (2006b). TLR signaling. Cell death and differentiation *13*, 816-825.

Kishimoto, T. (2006). Interleukin-6: discovery of a pleiotropic cytokine. Arthritis research & therapy *8 Suppl 2*, S2.

Klochendler, A., Weinberg-Corem, N., Moran, M., Swisa, A., Pochet, N., Savova, V., Vikesa, J., Van de Peer, Y., Brandeis, M., Regev, A.*, et al.* (2012). A transgenic mouse marking live replicating cells reveals in vivo transcriptional program of proliferation. Developmental cell *23*, 681-690.

Kopf, M., Baumann, H., Freer, G., Freudenberg, M., Lamers, M., Kishimoto, T., Zinkernagel, R., Bluethmann, H., and Kohler, G. (1994). Impaired immune and acute-phase responses in interleukin-6-deficient mice. Nature *368*, 339-342.

Kraakman, M.J., Kammoun, H.L., Allen, T.L., Deswaerte, V., Henstridge, D.C., Estevez, E., Matthews, V.B., Neill, B., White, D.A., Murphy, A.J.*, et al.* (2015). Blocking IL-6 trans-signaling prevents high-fat diet-induced adipose tissue macrophage recruitment but does not improve insulin resistance. Cell metabolism *21*, 403-416.

Kruger, T., Benke, D., Eitner, F., Lang, A., Wirtz, M., Hamilton-Williams, E.E., Engel, D., Giese, B., Muller-Newen, G., Floege, J.*, et al.* (2004). Identification and functional characterization of dendritic cells in the healthy murine kidney and in experimental glomerulonephritis. J Am Soc Nephrol *15*, 613-621.

Ladel, C.H., Blum, C., Dreher, A., Reifenberg, K., Kopf, M., and Kaufmann, S.H. (1997). Lethal tuberculosis in interleukin-6-deficient mutant mice. Infect Immun *65*, 4843-4849.

Lee, S., Huen, S., Nishio, H., Nishio, S., Lee, H.K., Choi, B.S., Ruhrberg, C., and Cantley, L.G. (2011). Distinct macrophage phenotypes contribute to kidney injury and repair. J Am Soc Nephrol *22*, 317-326.

Lee, S.O., Lou, W., Hou, M., de Miguel, F., Gerber, L., and Gao, A.C. (2003). Interleukin-6 promotes androgen-independent growth in LNCaP human prostate cancer cells. Clinical cancer research : an official journal of the American Association for Cancer Research *9*, 370-376.

Lu, J., Cao, Q., Zheng, D., Sun, Y., Wang, C., Yu, X., Wang, Y., Lee, V.W., Zheng, G., Tan, T.K.*, et al.* (2013). Discrete functions of M2a and M2c macrophage subsets determine their relative efficacy in treating chronic kidney disease. Kidney Int *84*, 745-755.

Marin, V., Montero-Julian, F., Gres, S., Bongrand, P., Farnarier, C., and Kaplanski, G. (2002). Chemotactic agents induce IL-6Ralpha shedding from polymorphonuclear cells: involvement of a metalloproteinase of the TNF-alpha-converting enzyme (TACE) type. Eur J Immunol *32*, 2965-2970.

Martinez, J.J., Mulvey, M.A., Schilling, J.D., Pinkner, J.S., and Hultgren, S.J. (2000). Type 1 pilus-mediated bacterial invasion of bladder epithelial cells. The EMBO journal *19*, 2803-2812.

Matsumoto, K. (1990). Production of interleukin-1 by glomerular macrophages in nephrotoxic serum nephritis. American journal of nephrology *10*, 502-506.

McGrath, K.E., Frame, J.M., Fegan, K.H., Bowen, J.R., Conway, S.J., Catherman, S.C., Kingsley, P.D., Koniski, A.D., and Palis, J. (2015a). Distinct Sources of Hematopoietic Progenitors Emerge before HSCs and Provide Functional Blood Cells in the Mammalian Embryo. Cell Rep *11*, 1892-1904.

McGrath, K.E., Frame, J.M., and Palis, J. (2015b). Early hematopoiesis and macrophage development. Semin Immunol *27*, 379-387.

McGrath, K.E., and Palis, J. (2005). Hematopoiesis in the yolk sac: more than meets the eye. Exp Hematol *33*, 1021-1028.

Miyazaki, J., Kawai, K., Oikawa, T., Johraku, A., Hattori, K., Shimazui, T., and Akaza, H. (2006). Uroepithelial cells can directly respond to Mycobacterium bovis bacillus Calmette-Guerin through Toll-like receptor signalling. BJU international *97*, 860-864.

Moore, P.S., Boshoff, C., Weiss, R.A., and Chang, Y. (1996). Molecular mimicry of human cytokine and cytokine response pathway genes by KSHV. Science *274*, 1739-1744.

Moriel, D.G., Heras, B., Paxman, J.J., Lo, A.W., Tan, L., Sullivan, M.J., Dando, S.J., Beatson, S.A., Ulett, G.C., and Schembri, M.A. (2016). Molecular and Structural Characterization of a Novel Escherichia coli Interleukin Receptor Mimic Protein. mBio *7*, e02046.

Motwani, M.P., and Gilroy, D.W. (2015). Macrophage development and polarization in chronic inflammation. Semin Immunol *27*, 257-266.

Mroczko, B., Groblewska, M., Gryko, M., Kedra, B., and Szmitkowski, M. (2010). Diagnostic usefulness of serum interleukin 6 (IL-6) and C-reactive protein (CRP) in the differentiation between pancreatic cancer and chronic pancreatitis. Journal of clinical laboratory analysis *24*, 256-261.

Mulvey, M.A., Lopez-Boado, Y.S., Wilson, C.L., Roth, R., Parks, W.C., Heuser, J., and Hultgren, S.J. (1998). Induction and evasion of host defenses by type 1-piliated uropathogenic Escherichia coli. Science *282*, 1494-1497.

Mulvey, M.A., Schilling, J.D., and Hultgren, S.J. (2001). Establishment of a persistent Escherichia coli reservoir during the acute phase of a bladder infection. Infect Immun *69*, 4572-4579.

Mulvey, M.A., Schilling, J.D., Martinez, J.J., and Hultgren, S.J. (2000a). Bad bugs and beleaguered bladders: interplay between uropathogenic Escherichia coli and innate host defenses. Proc Natl Acad Sci *97*, 8829-8835.

Mulvey, M.A., Schilling, J.D., Martinez, J.J., and Hultgren, S.J. (2000b). Bad bugs and beleaguered bladders: interplay between uropathogenic Escherichia coli and innate host defenses. Proc Natl Acad Sci U S A *97*, 8829-8835.

Murray, P.J., Allen, J.E., Biswas, S.K., Fisher, E.A., Gilroy, D.W., Goerdt, S., Gordon, S., Hamilton, J.A., Ivashkiv, L.B., Lawrence, T.*, et al.* (2014). Macrophage activation and polarization: nomenclature and experimental guidelines. Immunity *41*, 14-20.

Murray, P.J., and Wynn, T.A. (2011). Protective and pathogenic functions of macrophage subsets. Nat Rev Immunol *11*, 723-737.

Nishimoto, N., and Kishimoto, T. (2006). Interleukin 6: from bench to bedside. Nature clinical practice Rheumatology *2*, 619-626.

Oldenburg, M., Kruger, A., Ferstl, R., Kaufmann, A., Nees, G., Sigmund, A., Bathke, B., Lauterbach, H., Suter, M., Dreher, S.*, et al.* (2012). TLR13 recognizes bacterial 23S rRNA devoid of erythromycin resistance-forming modification. Science *337*, 1111-1115.

Panichi, V., Maggiore, U., Taccola, D., Migliori, M., Rizza, G.M., Consani, C., Bertini, A., Sposini, S., Perez-Garcia, R., Rindi, P.*, et al.* (2004). Interleukin-6 is a stronger predictor of total and cardiovascular mortality than C-reactive protein in haemodialysis patients. Nephrol Dial Transplant *19*, 1154-1160.

Perdiguero, E.G., Klapproth, K., Schulz, C., Busch, K., Azzoni, E., Crozet, L., Garner, H., Trouillet, C., de Bruijn, M.F., Geissmann, F.*, et al.* (2014). Tissue-resident macrophages originate from yolk-sac-derived erythro-myeloid progenitors. Nature.

Robbins, C.S., Hilgendorf, I., Weber, G.F., Theurl, I., Iwamoto, Y., Figueiredo, J.L., Gorbatov, R., Sukhova, G.K., Gerhardt, L.M., Smyth, D.*, et al.* (2013). Local proliferation dominates lesional macrophage accumulation in atherosclerosis. Nat Med *19*, 1166-1172.

Rogers, N.M., Ferenbach, D.A., Isenberg, J.S., Thomson, A.W., and Hughes, J. (2014). Dendritic cells and macrophages in the kidney: a spectrum of good and evil. Nature reviews Nephrology *10*, 625-643.

Romani, L., Mencacci, A., Cenci, E., Spaccapelo, R., Toniatti, C., Puccetti, P., Bistoni, F., and Poli, V. (1996). Impaired neutrophil response and CD4+ T helper cell 1 development in interleukin 6-deficient mice infected with Candida albicans. The Journal of experimental medicine *183*, 1345-1355.

Rose-John, S. (2012). IL-6 trans-signaling via the soluble IL-6 receptor: importance for the pro-inflammatory activities of IL-6. Int J Biol Sci *8*, 1237-1247.

Rose-John, S., and Heinrich, P.C. (1994). Soluble receptors for cytokines and growth factors: generation and biological function. Biochem J *300 (Pt 2)*, 281-290.

Russo, T.A., and Johnson, J.R. (2003). Medical and economic impact of extraintestinal infections due to Escherichia coli: focus on an increasingly important endemic problem. Microbes and infection / Institut Pasteur *5*, 449-456.

Sakakibara, S., and Tosato, G. (2011). Viral interleukin-6: role in Kaposi's sarcoma-associated herpesvirus: associated malignancies. Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research *31*, 791-801.

Sansone, P., Storci, G., Tavolari, S., Guarnieri, T., Giovannini, C., Taffurelli, M., Ceccarelli, C., Santini, D., Paterini, P., Marcu, K.B.*, et al.* (2007). IL-6 triggers malignant features in mammospheres from human ductal breast carcinoma and normal mammary gland. J Clin Invest *117*, 3988-4002.

Savill, J., and Fadok, V. (2000). Corpse clearance defines the meaning of cell death. Nature *407*, 784-788.

Scheller, J., Chalaris, A., Schmidt-Arras, D., and Rose-John, S. (2011). The pro- and anti-inflammatory properties of the cytokine interleukin-6. Biochimica et biophysica acta *1813*, 878-888.

Schilling, J.D., Lorenz, R.G., and Hultgren, S.J. (2002). Effect of trimethoprim-sulfamethoxazole on recurrent bacteriuria and bacterial persistence in mice infected with uropathogenic Escherichia coli. Infect Immun *70*, 7042-7049.

Schiwon, M., Weisheit, C., Franken, L., Gutweiler, S., Dixit, A., Meyer-Schwesinger, C., Pohl, J.M., Maurice, N.J., Thiebes, S., Lorenz, K.*, et al.* (2014). Crosstalk between Sentinel and Helper Macrophages Permits Neutrophil Migration into Infected Uroepithelium. Cell *156*, 456-468.

Schulz, C., Gomez Perdiguero, E., Chorro, L., Szabo-Rogers, H., Cagnard, N., Kierdorf, K., Prinz, M., Wu, B., Jacobsen, S.E., Pollard, J.W.*, et al.* (2012). A lineage of myeloid cells independent of Myb and hematopoietic stem cells. Science *336*, 86-90.

Senaldi, G., Varnum, B.C., Sarmiento, U., Starnes, C., Lile, J., Scully, S., Guo, J., Elliott, G., McNinch, J., Shaklee, C.L.*, et al.* (1999). Novel neurotrophin-1/B cell-stimulating factor-3: a cytokine of the IL-6 family. Proc Natl Acad Sci U S A *96*, 11458-11463.

Serbina, N.V., and Pamer, E.G. (2006). Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2. Nat Immunol *7*, 311-317.

Shi, C., Hohl, T.M., Leiner, I., Equinda, M.J., Fan, X., and Pamer, E.G. (2011). Ly6G+ neutrophils are dispensable for defense against systemic Listeria monocytogenes infection. Journal of immunology *187*, 5293-5298.

Shi, C., and Pamer, E.G. (2011). Monocyte recruitment during infection and inflammation. Nat Rev Immunol *11*, 762-774.

Skiniotis, G., Boulanger, M.J., Garcia, K.C., and Walz, T. (2005). Signaling conformations of the tall cytokine receptor gp130 when in complex with IL-6 and IL-6 receptor. Nature structural & molecular biology *12*, 545-551.

Steinman, R.M., and Cohn, Z.A. (1973). Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. J Ex Med *137*, 1142-1162.

Tipping, P.G., Leong, T.W., and Holdsworth, S.R. (1991). Tumor necrosis factor production by glomerular macrophages in anti-glomerular basement membrane glomerulonephritis in rabbits. Lab Invest *65*, 272-279.

Tittel, A.P., Heuser, C., Ohliger, C., Knolle, P.A., Engel, D.R., and Kurts, C. (2011). Kidney dendritic cells induce innate immunity against bacterial pyelonephritis. J Am Soc Nephrol *22*, 1435-1441.

Trikha, M., Corringham, R., Klein, B., and Rossi, J.F. (2003). Targeted anti-interleukin-6 monoclonal antibody therapy for cancer: a review of the rationale and clinical evidence. Clinical cancer research : an official journal of the American Association for Cancer Research *9*, 4653-4665.

van Furth, R., and Cohn, Z.A. (1968). The origin and kinetics of mononuclear phagocytes. The Journal of experimental medicine *128*, 415-435.

Wang, Y., and Harris, D.C. (2011). Macrophages in renal disease. J Am Soc Nephrol *22*, 21-27.

Wang, Y., Wang, Y., Cao, Q., Zheng, G., Lee, V.W., Zheng, D., Li, X., Tan, T.K., and Harris, D.C. (2008). By homing to the kidney, activated macrophages potently exacerbate renal injury. Am J Pathol *172*, 1491-1499.

Weidle, U.H., Klostermann, S., Eggle, D., and Kruger, A. (2010). Interleukin 6/interleukin 6 receptor interaction and its role as a therapeutic target for treatment of cachexia and cancer. Cancer genomics & proteomics *7*, 287-302.

Weisheit, C.K., Engel, D.R., and Kurts, C. (2015). Dendritic Cells and Macrophages: Sentinels in the Kidney. Clinical journal of the American Society of Nephrology : CJASN *10*, 1841-1851.

Yang, J., Zhang, L., Yu, C., Yang, X.F., and Wang, H. (2014). Monocyte and macrophage differentiation: circulation inflammatory monocyte as biomarker for inflammatory diseases. Biomarker research *2*, 1.

Yasukawa, K., Hirano, T., Watanabe, Y., Muratani, K., Matsuda, T., Nakai, S., and Kishimoto, T. (1987). Structure and expression of human B cell stimulatory factor-2 (BSF-2/IL-6) gene. The EMBO journal *6*, 2939-2945.

Yona, S., Kim, K.W., Wolf, Y., Mildner, A., Varol, D., Breker, M., Strauss-Ayali, D., Viukov, S., Guilliams, M., Misharin, A.*, et al.* (2013). Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. Immunity *38*, 79-91.

Zhang, D., Zhang, G., Hayden, M.S., Greenblatt, M.B., Bussey, C., Flavell, R.A., and Ghosh, S. (2004). A toll-like receptor that prevents infection by uropathogenic bacteria. Science *303*, 1522-1526.

Erklärung:

Hiermit erkläre ich, gem. § 7 Abs. (2) d) + f) der Promotionsordnung der Fakultät für Biologie zur Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient, bei der Abfassung der Dissertation nur die angegeben Hilfsmittel benutzt und alle wörtlich oder inhaltlich übernommenen Stellen als solche gekennzeichnet habe.

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Hiermit erkläre ich, gem. § 6 Abs. 2, g der Promotionsordnung der Fakultät für Biologie zur Erlangung der Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema *„**Local proliferation of Ly6C+ monocytes in inflamed tissues and during bacterial infections'** Titel der Dissertation"* zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von *(Akanksha Dixit)* befürworte.

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Prof. Dr. Daniel Engel	**Prof. Dr. Daniel Engel**
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