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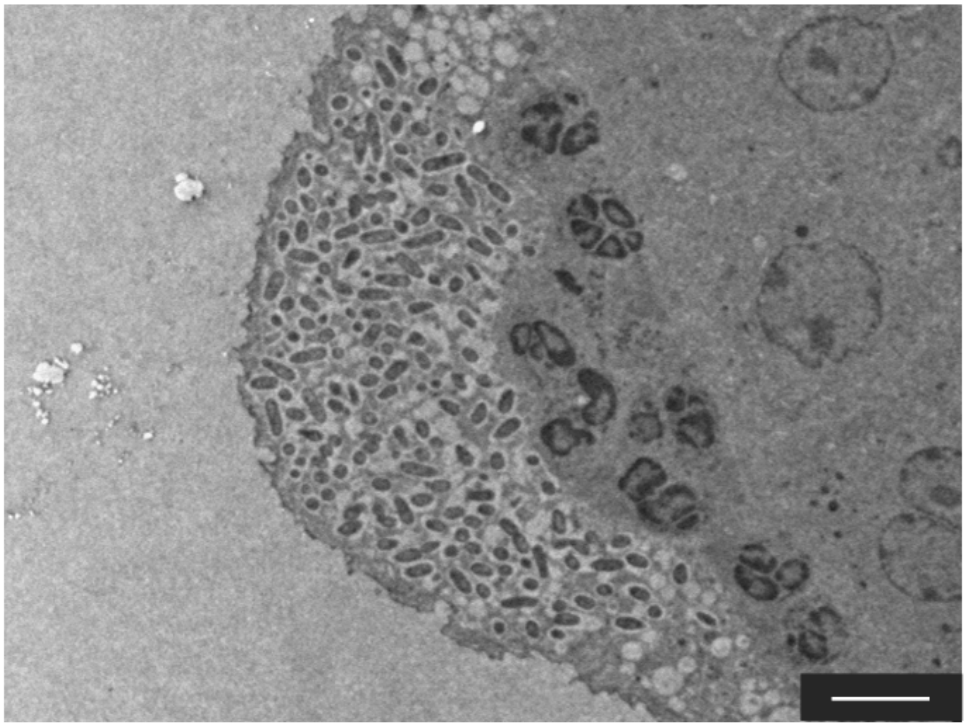
in bacterial urinary tract infection



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The innate cellular immune response in bacterial urinary tract infection

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1. SUMMARY

Urinary tract infections (UTI), such as cystitis or pyelonephritis, affect a large proportion of the world population and most of these infections are caused by UPEC bearing distinct virulence factors. Immune mediators established to be of importance for innate immune defense against UPEC include antimicrobial substances such as reactive oxygen species, exfoliation of uroepithelial cells and infiltration of neutrophil granulocytes (NΦG). In many infections, also macrophages (MΦ) and dendritic cells (DC) have been shown to be recruited by various chemokine receptors to sites of infection. Recruitment and functional role of these cells in UTI, however, are unknown. The present study aimed at answering these open questions.

To this end, a murine infection model facilitated by instillation of UPEC into the bladder was established. Vesical infection caused recruitment of large numbers of NΦG, DC and MΦ to the bladder submucosa and to the uroepithelium. Most DC exhibited the $CD11b^{+}$ $F4/80^{+}$ $CD8\alpha^{-}$ $Gr1^{-}$subtype. In addition, also CCR2-dependent $CD11b^{INT}$ DC producing iNOS and TNFα (Tip-DC) were detected, which have previously been shown to be crucial for the defense against other bacterial infections. However, CCR2-deficient mice that lacked the Tip-DC subset showed unimpaired clearance of UPEC from the infected bladder. Also mice devoid of DC, including the Tip-DC subset, remained capable of effective innate clearance of UPEC. However, DC were found to be efficient at phagocytosis of UPEC. This uptake strongly correlated with expression of MHC class II and costimulatory molecules, which were dependent on CCR2. These findings demonstrate that DC are dispensable for innate immunity against UPEC, but suggest a role in induction of adaptive immunity.

In addition to DC, two subsets of MΦ, CX_3CR1-expressing $Gr1^{LO}$ MΦ and CCR2-expressing $Gr1^{HI}$ MΦ, were found in infected bladders. These subsets have been described in other infections, but it remained unresolved whether they exerted distinct immune effector functions. In UTI, only $Gr1^{LO}$ MΦ produced iNOS, whereas $Gr1^{HI}$ MΦ effectively produced TNFα. All phagocytes examined produced ß1 defensins and not only epithelial cells as previously proposed. Interestingly, NΦG were most active at phagocytosis of UPEC. These findings are the first to reveal functional differences between $Gr1^{LO}$ and $Gr1^{HI}$ MΦ in infection.

Moreover, the factors responsible for egress and immigration of MΦ in UTI were identified using a recently described *in vivo* labeling technique. Finally, CCR2 contributed to the abundance of monocytes in the blood and the bladder and may affected the release of monocytes from the bone marrow (BM) into the circulation.

In conclusion, the present study revealed mechanisms important in recruitment, migration and for expression of effector functions by different phagocyte cell types against bacterial infections. These findings advance our understanding of immunity against bacterial infections and extended the molecular mechanism important for egress and immigration of monocytes throughout the organism.

2. TABLE OF CONTENT

3. ABBREVIATIONS

APC	Antigen Presenting Cell
BM	Bone marrow
CCL	Chemokine Ligand
CCR	Chemokine Receptor
CDC	Conventional DC
CFU	Colony Forming Unit
Clo-lip	Clodronate-Liposomes
CPS	*E.coli* Proteus Streptococcus
DC	Dendritic Cell
DT	Diphteria Toxin
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetra-acetic acid
EGF	Epidermal Growth Factor
EM	Electron Microscopy
FCS	Foetal Calf Serum
GFP	Green Fluorescent Protein
Het	Heterozygeous
ICAM-1	Intracellular Adhesion Molecule
IF	Immunofluorescence
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
iNOS	inducible Nitric Oxide Synthetase
LC	Langerhans cell
LN	Lymph Node
Lx	Latex

M	Molar
MCP-1	Macrophage Chemoattractant Protein-1
M-CSF	Macrophage- Colony Stimulating Factor
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
MIP-2	Macrophage Inflammatory Protein-2
mL	milli Liter
mM	milli Molar
MΦ	Macrophage
ng	nano Gramm
NO	Nitric oxide
NΦG	Neutrophil Granulocyte
pDC	plasmacytoid DC
PMN	Polymorphnuclear cells
RANTES	Regulated on Activation, Normal T-cell Expressed and Secreted
RCB	Red blood cell removal buffer
TGF-ß	Transforming Growth Factor - beta
Tip-DC	TNFα iNOS producing – Dendritic Cell
TLR	Toll Like Receptor
TNF	Tumor Necrosis Factor
UPEC	Uropathogenic *E.coli*
UTI	Urinary Tract Infection
VCAM-1	Vascular Cell Adhesion Molecule-1

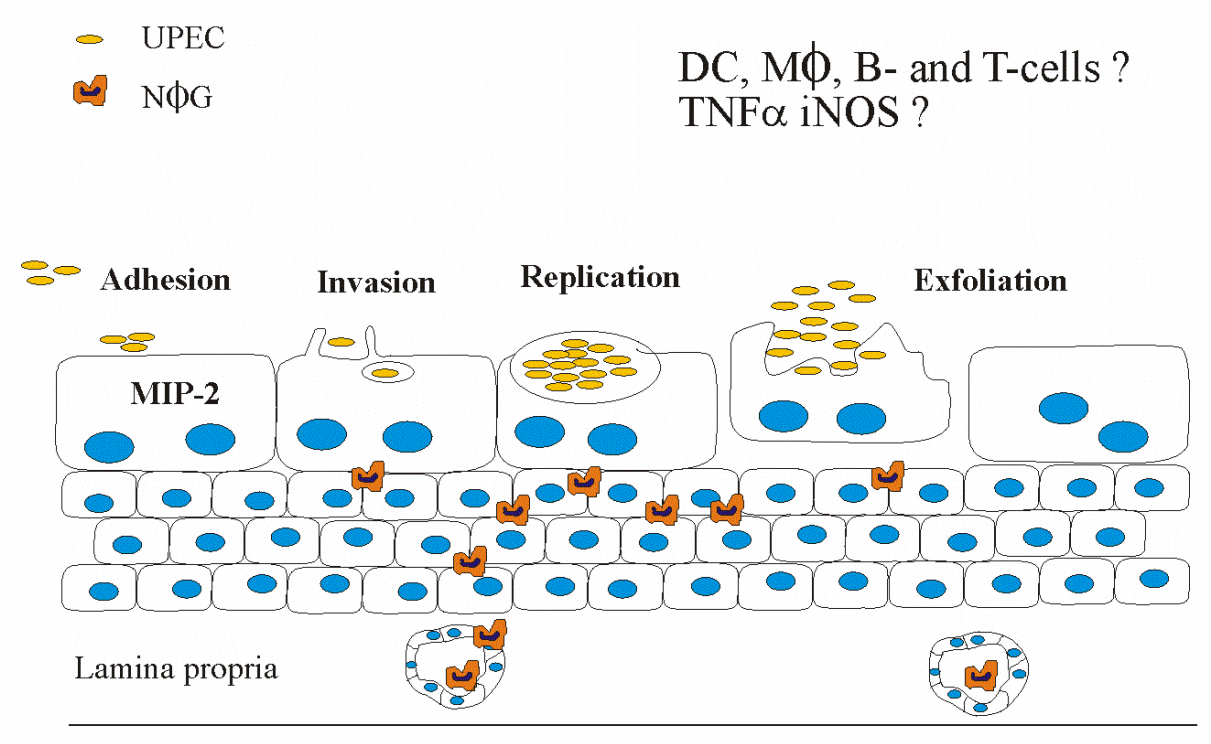
4. INTRODUCTION

4.1. UPEC in UTI

UTI, such as cystitis or pyelonephritis, are among the most prevalent infections and account for significant morbidity and medical costs in developed countries (36). Most of these infections are caused by UPEC bearing distinct virulence factors, such as fimbriae or pili. These factors can be monomers, simple oligomers or supramolecular fibers (43) and facilitate bacterial attachment to uroepithelial cells, bacterial ascension from the bladder to the kidney and organ infiltration (54).

4.2. Defense mechanisms of the infected host

Various defense mechanisms protect the body from UTI. Uroepithelial cells secrete antimicrobial agents (79), or are exfoliated when infected by UPEC (54). Among the immune cells involved in the defense against UTI, NΦG play a crucial role (27). These non-resident cells are attracted by mediators such as CXCL8 (MIP-2) (32) or TNFα (48). Sensing of bacteria by immune cells can occur by toll-like receptors (TLR) (83). In particular, TLR4 recognizes LPS in gram-negative bacteria (65), while TLR11 responded to unidentified molecular patterns unique to UPEC (94). Recently, it has been shown that TLR4 expression both in uroepithelial and in hematopoietic cells within the bladder was important for defense against UTI (75). The identity of the TLR4-expressing hematopoietic cells remained unresolved. Possible candidates known to express TLR are MΦ and DC. The current model which described the interplay between the host cells and the invading UPEC is shown in Scheme 1. UPEC adhere, invade and replicate in uroepithelial cells. Afterwards these infected cells can exfoliate and release UPEC into the bladder lumen. NΦG are known to infiltrate the uroepithelium to control the infection. The role of DC, MΦ, B- and T-cells and the source of several innate effector molecules in this infection are unknown.



Scheme 1. Interplay between UPEC and host defense mechanisms

4.3. Crosstalk between NΦG and mononuclear cells

NΦG are considered as non antigen-specific leukocytes, involved in the first line of defense against infection. Their functions in infection include phagocytosis and secretion of preformed enzymes and partially reduced molecules of oxygen. In addition to these direct anti-microbial functions, NΦG contribute to the recruitment, activation and programming of APC by secreting proinflammatory chemotactic signals that attract monocytes and DC (13). TNFα is proposed to be one of the major NΦG-derived chemokines that drive activation and differentiation of MΦ and DC (89) . Furthermore, there is cross-talk of NΦG with MΦ resulting in the generation of pro- or anti- inflammatory MΦ. Their interaction with DC promotes maturation of the latter (7). NΦG also proteolytically cleave prochemerin to generate chemerin, one of the few chemokines that attracts both immature DC and pDC (92). This activation and differentiation of cells by NΦG is bidirectional. NΦG function and activation can be altered by signals from MΦ and DC (20).

4.4. DC in UTI

DC originate from a hematopoietic stem cell precursor and reside in lymphoid and non-lymphoid tissues. They are specialized at uptake, transport, processing and presentation of antigen to T lymphocytes and represent the most effective inducers of adaptive immunity (51). Several murine DC subsets have been identified that differ in surface phenotype, activation state and functionality. There are three major DC subtypes: epidermal Langerhans cells (LC), plasmacytoid DC (pDC) and conventional DC (cDC) (4, 5, 78) (6). These DC subsets can be subdivided by defining their cell surface molecules. Langerin is a characteristic molecule for LC whereas B220 defines the pDC subset. CDC can be subdivided into $CD11b^{-}\,CD8\alpha^{+}$ and $CD11b^{+}\,CD8\alpha^{-}$ DC. $CD8\alpha^{+}$ DC have been shown to exhibit cross presenting ability important for activation of $CD8\alpha^{+}$ T cells (18). In the blood, the spleen and in non-lymphoid tissues, cDC possess a more immature phenotype, characterized by low levels of MHC class II and costimulatory molecules, whereas cDC in the lymph nodes have a more mature phenotype characterized by high levels of these molecules. As the spleen lacks afferent lymphatic vessels, it is likely that the immature cDC in the spleen are blood-derived, whereas mature cDC in the lymph node originate at least partially from tissue-derived DC, which matured after antigen encounter. In addition to the well-described role of DC as inducers of adaptive immune responses, recent studies have proposed a role of DC also in innate immunity (68). For example, pDC stimulated antiviral defense by secretion of type I interferons (15). In bacterial infections, a newly discovered DC subpopulation, called “Tip-DC”, which produce TNFα and iNOS has been reported to be critical in the defense against the gram-positive rod *Listeria monocytogenes* (77). These cells were absent in CCR2-deficient mice, and the resulting lack of splenic iNOS- and TNFα-producing cells led to susceptibility to these gram-positive bacteria. Although these two mediators are known to be generally involved in antibacterial defense, their roles in UTI, as those of CCR2 and of Tip-DC are unknown.

4.5. Chemokines involved in homeostatic and inflammatory migration of leukocytes

Chemokines are small proteins that act as activators and chemoattractants for leukocyte subpopulations. They comprise one of two groups of chemoattractants, the other one being the ‘‘classical’’ chemoattractants, bacterial-derived N-formyl peptides, complement fragment peptides C5a and C3a as well as lipid molecules such as leukotriene B4 and platelet-activating factors (25, 28, 31, 74). The chemokine superfamily can be divided into 4 groups (CXC, CX3C, CC, and C) according to the positioning of the first 2 closely paired and highly conserved cysteins of the amino acid sequence (Table 1). Their chemotactic function is mediated through a family of 7-transmembrane-spanning G-protein–coupled receptors, the chemokine receptors (CCR).

Receptor	Ligands	Cellular distribution
CXC chemokines		
CXCR2	GRO (KC), LIX (GCP2), MIP-2	N, M, T, NK, En
CXCR3	MIG, crg-2 (IP-10); ITAC (CXCL11)	act T
CXCR4	SDF-1 (PBSF)	T, B, DC, En
CXCR5	BLC (BCA-1)	B
CC chemokines		
CCR1	MIP-1a, RANTES, MCP-3	N, M, T, NK, B
CCR2	MCP-1, MCP-3, MCP-5	M, DC
CCR3	Eotaxin, RANTES, MIP-1a	Eo, Bs, T
CCR4	MDC (ABCD-1), TARC	act T, NK T, DC, reg T
CCR5	RANTES, MIP-1a, MIP-1b	T, M, DC
CCR6	MIP-3a (LARC, Exodus)	T, B, DC
CCR7	SLC (6Ckine), ELC (MIP-3b)	T, B, DC
CCR8	TCA-3 (I-309)	M, Thymus
CCR9	TECK	T, Thymus
C chemokine		
XCR1	Lymphotactin (SCM-1)	T, B, NK
CX3C chemokine		
CX_3CR1	Fraktalkine (neurotactin)	act T, NK, M, DC

act T, activated T cell; B, B-cell; DC, Dendritic cell; En, Endothelial cell; M, monocyte/MΦ
N, neutrophil; NK, natural killer cell; reg T, regulatory T cell;T, T-cell

Table 1. Chemokines, their ligands and the cellular distribution.

Interaction of the chemokine ligand with its receptor causes attraction of various cell types into non-inflamed and inflamed tissue. Homeostatic chemokines like fraktalkine induce constitutive migration of immune cells into tissue under non-inflammatory conditions, whereas inflammatory cytokines like MCP-1 and MIP-2 attract MΦ and NΦG into inflamed tissue. In models of bacterial respiratory tract and skin infections, recruitment of leukocytes, in particular NΦG, MΦ and DC from the circulation is essential to induce innate immune responses in order to control the pathogens (50) (62) (73). The migration of NΦG is critically dependent on the chemokine MIP-2 and TNFα. It has been shown that in the absence of MIP-2, UTI is aggravated and increased renal scarring was observed (23). Inflammatory MΦ and DC express the chemokine receptors CCR1, CCR2 and CCR5 on their surface that enable migration into inflamed tissue (91). A role of CCR2 in UTI is supported by a study demonstrating the release of the CCR2 ligand, MCP-1 (CCL2), but the contribution of this receptor to the defense against UTI is unknown (30). Resident tissue MΦ and DC express the fraktalkine receptor CX_3CR1 on their surface. Although there is evidence that this receptor primarily mediates constitutive cellular migration into non-inflamed tissue, its exact function, in particular in inflammation, remains unknown.

4.6. CX_3CR1 mediates DC access to the intestinal lumen

DC and MΦ are critical in innate and adaptive immunity to the intestinal bacterial microbiota (58). DC populating the entire lamina propria of the small intestine were previously observed in mice (69) (49). These lamina propria DC were found to be dependent on the chemokine receptor CX_3CR1 to form transepithelial dendrites and furthermore, these protrusions enable DC to sample luminal antigens. CX_3CR1^+ DC were also found to contribute to the clearance of entero-invasive pathogens. (88) (57) (58).

CX_3CR1^+ DC have been found also within normal murine kidneys (80). Laser-scanning confocal microscopy revealed an extensive, contiguous network of stellate-shaped CX_3CR1^+ DC throughout the interstitial and mesangial spaces of the entire kidney. Intravital microscopy of the superficial cortex showed stationary interstitial CX_3CR1^+ DC that continually probed the surrounding tissue environment through dendrite extensions. Flow cytometry of renal CX_3CR1^+ DC showed significant expression of CD11c and F4/80, high major histocompatibility complex class II and FcR expression (80).

4.7. Mononuclear cells in the circulation arise from BM progenitors

Mononuclear leukocytes in the circulation develop from BM-derived progenitors, which express the characteristic monocyte surface molecules F4/80, CD115 and CD11b (21) (24) (82) (66) (29). Most of these monocytes in the BM express high levels of Gr1 ($Gr1^{HI}$). Adoptive transfer experiments showed that $Gr1^{HI}$ monocyte progenitors in the BM gave rise to $Gr1^{HI}$ monocytes in the blood, but not to the $Gr1^{LO}$ subset (24). However, more recent studies showed a potential of the $Gr1^{HI}$ monocyte subset in the BM to give rise to $Gr1^{LO}$ monocytes in the blood and to LC (26) (21). A short time before ending this thesis, it has been shown that CCR2 may contribute to the emigration of monocytes from the BM into the circulation (76). In support of this, CCR2-deficient mice showed increased numbers of $CCR2^{HI}$ $Gr1^{HI}$ monocytes in the BM under infectious conditions, whereas the number of these monocytes in the circulation was diminished (76).

4.8. Blood monocytes consist of two different subsets: CX_3CR1^+ and $CCR2^+$

Peripheral blood monocytes are a heterogeneous cell population and represent about 5 to 10% of blood leukocytes. Two main subsets of monocytes, defined by different expression levels of CCR2, CX_3CR1 and Gr1 have been described in mice and humans (24) (21). CX_3CR1^{LO} $Gr1^{HI}$ monocytes are large granular mononuclear cells of 10 to 14 µm diameter, while CX_3CR1^{HI} $Gr1^{LO}$ cells are smaller mononuclear cells with a diameter of 8 to 12 µm (24). The CX_3CR1^{HI} $CCR2^{LO}$ $Gr1^{LO}$ subset is thought to be primarily recruited into non-inflamed tissue, whereas $CCR2^{HI}$ CX_3CR1^{LO} $Gr1^{HI}$ blood monocytes are actively recruited into inflamed tissue, e.g. inflamed skin or peritoneum (24) (82), the inflamed retina (93) and infection with *Toxoplasma gondii* (70) or *Listeria monocytogenes* (82). In many models of acute inflammation, $Gr1^{HI}$ monocyte recruitment was critically dependent on CCR2 (10) (53), but not on CX_3CR1 (38), but a crucial role of CX_3CR1 in constitutive migration into non-inflamed tissue is unknown.

It has been shown that both monocyte subsets can be depleted by intravenous administration of clo-lip into mice (84) (Fig. 1). Interestingly, the $CCR2^{+}$ $Gr1^{HI}$ monocyte subset reconstituted the circulation already 24 hours after depletion, whereas the CX_3CR1^{+} $Gr1^{LO}$ remained absent for at least two days.

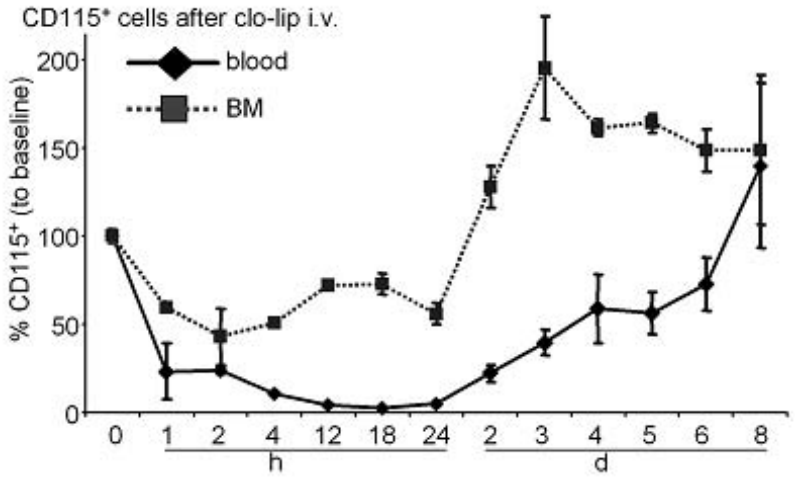


Fig. 1. Circulating monocytes after depletion (from (84))

4.9. Migratory fate of monocytes and their function as progenitors

Blood monocytes are thought to represent precursors for peripheral MΦ and DC (24). Once they emigrate across endothelial barriers, they differentiate into these different phagocyte phenotypes. The murine CX_3CR1^{LO} $Gr1^{HI}$ subset was termed "inflammatory monocyte" and represents circulating immediate precursors for antigen-presenting DC and $CD11c^{-}$ myeloid cells under inflammatory conditions. Furthermore, it has recently been shown that circulating $Gr1^{HI}$ monocytes give rise to the afore mentioned Tip-DC (76). Although stringent evidence is lacking, the CX_3CR1^{HI} $Gr1^{LO}$ murine subset has been termed "resident monocyte" and was proposed to represent a circulating precursor population which constitutively migrates into non-inflamed tissue. DC with innate immune functions (19) (77) have recently been shown to arise from $Gr1^{HI}$ monocytes (76). There is no experimental evidence that the other CX_3CR1^{HI} $Gr1^{LO}$ monocyte subset persisted longer in tissues and served as a precursor for resident myeloid cells in non-inflamed tissue (24). Less is known about the trafficking and fate of $Gr1^{LO}$ monocytes under inflammatory and non-inflammatory conditions. In contrast to $Gr1^{HI}$ monocytes, the $Gr1^{LO}$ counterpart rarely migrated into inflamed tissue, including the acutely inflamed peritoneum (24) (82) or the skin after

intracutaneous injection of Latex (Lx) beads (66), administration of vaccine formulations (45) or epicutaneous UV exposure (26). It has therefore been hypothesized that $Gr1^{LO}$ monocytes may fulfill critical roles in replacing resident MΦ or DC in the steady state (24). Given their higher surface expression of CX_3CR1 and the CX_3CL1-dependent transendothelial migration of human $CD16^+$ monocytes *in vitro* (1) (24), it has further been suggested that the chemokine receptor CX_3CR1 on $Gr1^{LO}$ monocytes is crucial for migration into non-inflamed tissue aimed at replacing resident MΦ or DC. However, the experimental evidence for the trafficking pattern and the potential role of $Gr1^{LO}$ monocytes as precursors for resident tissue cells is poor. After adoptive transfer, $Gr1^{LO}$ monocytes could be found in blood, spleen, liver, lung and brain of recipients for up to three days after transfer, and then at very diminished numbers by day 4 (24), but whether these adoptively transferred monocytes gave rise to DC is unknown.

Compelling experimental *in vivo* evidence for the ability of monocytes and MΦ to convert into DC is poor. After intradermal injection of fluorescently labeled Lx beads into the skin, mild inflammation is observed followed by the recruitment of phagocytic $CD11b^+$ monocytes. About 25% of these Lx-bearing monocytes migrated into the draining LN where they differentiated into $MHCII^+DEC205^+$ DC that expressed CD11c (67) (71). Surprisingly, neither CCR2 nor CX_3CR1 were essential for monocyte recruitment, migration or differentiation towards DC in this model (2). $Gr1^{LO}$ monocytes were not recruited at all in this model, but $Gr1^{HI}$ and a third minor subset of $Gr1^{INT}$ expressing monocytes, which appeared to represent the population that gave rise to DC. Furthermore, these cells expressed mRNA for CCR7 and CCR8, which are known to regulate the ability of DC to migrate into lymph nodes (66). It is clear that monocytes can give rise to DC under inflammatory conditions, but the turnover of DC in the steady state remains unresolved. Recently, a clonogenic BM progenitor that is $CD117^+$ (c-kit), $CD115^+$ and $CX3CR1^+$ has been identified that gave rise to monocytes, MΦ and resident $CD8\alpha^+$ spleen DC, which represent the DC subpopulation capable of cross-presentation (21). Direct evidence that a circulating monocyte subset gives rise to a resident peripheral DC population, was only provided for the differentiation of $Gr1^{HI}$ monocytes into epidermal LC (26), but experimental evidence for the $Gr1^{LO}$ subset to do so is limited.

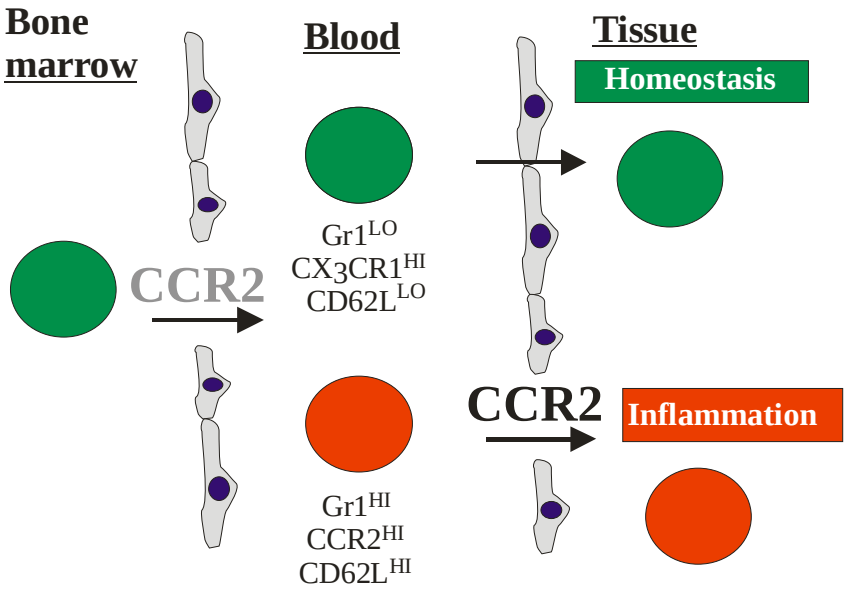
In the steady state, LC arise from a local precursor (53), which was dependent on CD115 expression (26). However, severe skin inflammation induced by local UV irradiation results in the full depletion of LC from skin, apparently including loss of the local precursor (53). In this setting, $Gr1^{HI}$, but not $Gr1^{LO}$ monocytes were recruited to the inflamed skin in a CCR2- and CCR6-dependent manner, proliferated locally and differentiated into functional LC or dermal MΦ *in vivo* (53) (52) (26). This work revealed the differentiation potential of monocytes and showed how they fulfilled their predicted role as circulating precursors for DC and MΦ, especially during inflammatory conditions, in which there is increased demand for DC and MΦ. Similarly, in the context of vaccination through skin and other mucosal routes, $Gr1^{HI}$ monocytes appear to be the most relevant precursors for cross-priming DC that initiate $CD8\alpha^{+}$ T cell responses.

Monocyte progenitors also contributed to the generation of DC with innate immune functions in infection models. In the spleen, $Gr1^{HI}$ monocytes have been linked to the formation of Tip-DC. Another interesting role for $Gr1^{HI}$ monocytes in the context of immunization is the ‘‘priming’’ of naive B cells that will ultimately facilitate their differentiation into Ig-producing cells. This process was recently found to be dependent on $Gr1^{HI}$ monocytes (37). Interestingly, these cells appeared to serve as the source for IL-4 that in turn triggered B cell priming (37).

4.10. Signals in the BM maintain the homeostasis of circulating leukocytes

Although several adhesion molecules and chemokines have been studied in the process of emigration from the BM, the underlying molecular mechanisms are not well understood. The chemokine SDF-1 has been shown to contribute to homing of progenitor cells to the BM (44). This chemokine was constitutively expressed on stromal cells (9, 56) and signaling to the receptor CXCR4 activated cell surface integrins (61). This interaction was essential for retention of precursor cells in the BM (72), whereas the lack leads to emigration of leukocytes out of the BM. Furthermore, very recent investigation by others have shown a contribution of CCR2 in the egress of BM progenitors into the circulation (76) (Scheme 2).

Monocytes, which are generated in the BM, are released into the circulating leukocyte pool (24). Expression of MCP-1, the ligand for CCR2 on these monocytes, is associated with migration of monocytes into inflamed tissue and therefore with immune defense against microbial pathogens (Scheme 2). CCR2-deficient mice showed increased susceptibilities to various pathogens, like *Listeria monocytogenes* (77) and diminished recruitment of monocytes to the site of infection (42). Although several studies showed expression of the CCR2 ligand MCP-1 in the bladder, the role of CCR2 in the recruitment of immune cells into non-infected and infected bladders remains unknown (87). Furthermore, signals that mediate and maintain homeostasis of monocytes in infected and non-infected mice are poorly understood (16).



Scheme 2. Monocyte subsets arise from a BM progenitor

5. AIMS OF THIS STUDY

The aim of the present study was to elucidate the recruitment mechanisms and the functionality of phagocytes in UTI, using a murine model of this infection facilitated by transurethral instillation of UPEC. In particular, the following specific questions were addressed:

1. Are DC recruited to the bladder under homeostasis and infectious conditions?

2. What is the role of DC, in particular Tip-DC in UTI, and which molecular mechanisms mediate their recruitment and their functionality in the bladder?

3. Are the composition and the number of MΦ and monocytes in organs involved in UTI, like the BM, the blood and the bladder changed?

4. Which molecular mechanism mediates the migration of MΦ, in particular of $Gr1^{LO}$ and $Gr1^{HI}$ MΦ under homeostasis and infectious conditions?

6. MATERIAL AND METHODS

6.1. Solutions, Media and Supplements

Red blood cell removal buffer (RCB) was prepared by dissolving 15.58g NH_4Cl (0.146 M), 0.074g ethylenediaminetetra-acetic acid (EDTA)-disodium salt, 2.0g $NaHCO_3$ in 2 litres of Milli Q water. The solution was autoclaved and the final pH of 7.3 was adjusted.

FACS buffer consisted of PBS containing 0.1% BSA and 0.1% NaN_3.

Wash buffer for the digested bladder cell suspensions consisted of HBSS without Ca^{2+} and Mg^{2+}, 10mM EDTA, 20mM Hepes and 0.5% BSA.

Wash buffer for BM cells consisted of DMEM, 5mM EDTA and 0.5% BSA.

Hanks complete consisted of HBSS containing 0.1% BSA and 5 mM EDTA

Foetal Calf Serum (FCS) was heat-inactivated by immersing in a 56°C waterbath for 30 minutes. The heat inactivated FCS was aliquoted and stored at -20°C.

Nylon mesh (30µm, 50µm, 100µm) to filtrate cell suspension was cut into small square pieces and autoclaved.

6.2. Mice and reagents

All mice used had been backcrossed >10 times to a C57BL/6 background and were bred and kept under SPF conditions. $CCR2^{-/-}$ $ApoE^{-/-}$ double knockout mice were kindly provided by C. Weber (Aachen, Germany) (14, 32), and crossed with C57BL/6 mice to yield CCR2-single knockout mice in the F2 generation. CD11c-DTR mice (10) expressing GFP and the diphtheria toxin receptor (DTR) on $CD11c^{+}$ cells had been backcrossed 10 times to the C57BL/6 background. To deplete DC, 4 ng diphtheria toxin per gram body weight was injected i.p. 72 hours and 24 hours before infection. CX_3CR1-deficient mice were kindly

provided by F. Geissmann (Paris, France) and D. Fogg (Paris, France). By replacing the CX_3CR1 allele, heterozygous and homozygous mice harbor green CX_3CR1 positive cells. Animal experiments had been approved by a local animal ethics reviewing board.

6.3. UPEC and UTI model

The UPEC strain 536 (O6:K15:H31) originated from a UTI patient (8) (12). The chromosomal integration of GFP into this UPEC strain was done by Dr. Ulrich Dobrindt, Würzburg. Tagging of *E. coli* 536 with a stable fluorescence marker was achieved by λ Red recombinase-mediated chromosomal insertion of a P*tet*$^{p/o}$::*gfp*mut3.1 fusion into the *attB* site of the bacteriophage λ (17). The promoterless *gfp*mut3.1 gene was amplified from pGFPmut3.1 (Clontech) by PCR using primers gfpfus1 (5'-GAATTAAA-GAGGAGAAATTAAG-3') and gfpfus2 (5'-CGCGGCAGCAAACGCCAGCCTGGCG-ATTCTCGAATCTGGCGACTGGCAGCGACTAGTAGGTCAGCTAATTAAGC-3'). The 98-bp *tet*$^{p/o}$ region was amplified from pASK75 (37) with primers tetfus1 (5'-TGAAATAGAAAAATGAATCCGTTGAAGCCTGCTTTTTTATACTAACTTGACCATC GAATGGCCAGATG-3') and tetfus2 (5'- CTTAATTTCTCCTCTTTAATATTTCACTTTT-CTCTATCACTGATAG-3'). Both PCR products were fused in a recombinant PCR with the primers tetfus1 and gfpfus2. The PCR resulted in amplification of an 850-bp P*tet*$^{p/o}$:*gfp*mut3.1 DNA fragment with flanking 50-bp overhangs homologous to the chromosomal bacteriophage λ attachment site *attB* which was subsequently cloned into pGEM-T Easy (Promega, Mannheim, Germany). This plasmid was used as template for further amplification of the 850-bp PCR fragment flanked by 50-bp overhangs homologous to the λ *attB* site with the primer pair tetfus1 and gfpfus2. The resulting PCR product was electroporated into *E. coli* 536/pKD46. Fluorescent derivatives of strain 536 with a chromosomally inserted P*tet*$^{p/o}$:*gfp*mut3.1 fusion were then selected using a Typhoon™ 8600 Variable Mode Imager (Molecular Dynamics; Krefeld, Germany).

To induce UTI, UPEC were grown overnight in LB medium, then harvested by centrifugation at 1200 g for 20´ and resuspended in PBS to a concentration of $1x10^{10}$ colony forming units (CFU) per mL. Anesthetized female mice of 8 to 10 wks of age were infected by transurethral inoculation of $5x10^8$ *E.coli 536* (0.05 mL) into the bladder using a soft polyethylene catheter (outer diameter 0.6 mm; BD, Heidelberg, Germany) (27) (54). To determine the bacterial load, the bladders were rinsed extensively with PBS *in situ*, then

removed under sterile conditions and homogenized mechanically. The number of bacteria was quantified by scoring CFU after overnight culture at 37°C on CPS ID plates (Biomerieux, Nürtingen, Germany).

6.4. Immunohistochemistry and electron microscopy

For immunohistology, organs were fixed with 0.1 M Tris pH 7.4, 0.05% Zinc-Acetate, 0.5 % Zinc-Chloride and embedded in steedman's wax (81). Blocks were cut into 5 µm sections and mounted on poly-L-lysin-coated glass slides. For identification of $CD11c^+$ cells, biotinylated anti-CD11c (clone HL-3, Pharmingen, Heidelberg, Germany) or isotype controls were used, revealed with Vectastain ABC kit (Vector Laboratories, Burlingame, CA) and 3,3'-diaminobenzidine (DAB) and counterstained with methyl green. For immunofluorescence, antibodies were revealed with Streptavidine-Alexa568 and counterstained with Hoechst 33258 (Molecular Probes, Karlsruhe, Germany).

For electron microscopy, bladder tissue was fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) followed by 2 % osmiumtetroxide (OsO_4). Tissues were embedded in epoxon 812 (Serva, Heidelberg, Germany) and 40-50 nm sections were cut with an LKB ultramicrotome UM IV (Leika, Frankfurt/Main, Germany) and analyzed using a CM10 Electron microscope (Philips, Hamburg, Germany).

6.5. Isolation and analysis of leukocytes from the bladder

A protocol for DC isolation from the kidney (41) was adapted for application to bladder tissue. Briefly, bladders were sliced with a scalpel and digested for 30 min at 37°C with 0,5 mg/mL collagenase (Sigma Aldrich, Taufkirchen, Germany) and 100 µg/mL DNAse I (Sigma Aldrich, Taufkirchen, Germany) in RPMI 1640 medium (Invitrogen, Karlsruhe, Germany) containing 0.5% FCS (PAA Laboratories, Pasching, Austria) and 20 mM HEPES. Cell suspensions were filtered through 100 µm nylon mesh and washed with HBSS without Ca^{2+} and Mg^{2+} containing 10 mM EDTA, 0.1% BSA and 20 mM HEPES. The number of viable cells was determined by trypan blue staining.

6.6. Isolation and analysis of leukocytes from the blood and BM

Whole blood was subjected to red cell lysis using RCB and washed twice in DMEM (Invitrogen, Karlsruhe, Germany) containing 5 mM EDTA and 0.5% BSA. Cells were washed in HBSS (Invitrogen, Karlsruhe, Germany) containing 0.02% NaN_3 and 2 mM EDTA and 2% FCS.

BM cells were obtained by flushing the femur with HBSS containing 0.1% BSA and 5 mM EDTA followed by red blood lysis and two washing steps in HBSS (Invitrogen, Karlsruhe, Germany).

6.7. Depletion of monocytes

Clodronate or PBS was incorporated into liposomes as described previously (90) and blood monocytes were eliminated with these liposomes by i.v. injection of 0.25 ml into the lateral tail vein (82). Control liposomes that incorporated PBS instead of clodronate were used, but without affecting total monocytes or subpopulations.

6.8. Labeling of blood monocytes

0.5-µm FITC-conjugated (yellow gold) plain microspheres (2.5% solids; Polysciences Inc., Heidelberg, Germany) were diluted 1:25 in PBS, and 250 µl of the solution was injected into the lateral tail vein for labeling of $Gr1^{LO}$ monocytes. To label $Gr1^{HI}$ monocytes, 250 µl of liposomes containing clodronate were i.v. injected followed by 250 µl of fluorescent microspheres i.v. 16–18 hours later. Blood monocytes were fully depleted by clo-lip administration (82), but splenic and liver MΦ are also eliminate (90). Other MΦ populations are protected because clo-lip will not cross vascular barriers (90).

6.9. Flow cytometrical analysis

F_c receptors were always blocked with 24G2 culture supernatant or with a combination of human, mouse and rabbit serum. Titrated amounts of the following labeled antibodies from Pharmingen (Heidelberg, Germany) were used for staining of $1x10^6$ cell samples: anti MHC class II-FITC (25-9-3), anti iNOS-FITC (BD6), anti-TNFα APC, anti CD80-biotin (16-10A1), anti CD86-FITC (GL1), anti CD40-APC (3/23), anti CD11b-PerCp-Cy5.5 (M1/70), anti Gr1-PE/Cy7 (RB6-8C5) and anti CD11c-APC (HL-3). Anti F4/80-biotin (CI:A3-1) and MIP-2 biotin (AAM48B) was from Serotec (Düsseldorf, Germany), CD115 PE (AFS98) was from eBioscience (San Diego, USA) and anti CCR2 (MC21) was kindly provided by Dr. M. Mack, Regensburg, Germany (47). Cells were analyzed on a LSR II cytometer (BD, Heidelberg, Germany) using Flow Jo software (Tristar, Phoenix, AR, USA). Forward and side scatter gating was adapted to include MΦ and NΦG. The abundance of different cell populations was calculated by adding 10µm PerCP-Cy5.5- or APC-labeled microbeads (BD, Heidelberg, Germany) into the tubes.

7. RESULTS

7.1. Different leukocytes are recruited into the bladder after infection

To study the role of immune cell subsets in UTI, UPEC were transurethrally instilled into the bladders of C57BL/6 mice. To quantitatively assess the extent of infection, the number of bacterial colony forming units (CFU) in the bladder was determined. After instillation of $5x10^8$ UPEC into the bladder, number of CFU decreased rapidly and 3 days after infection only few bacteria remained detectable in the bladder (Fig. 2A).

To correlate the bacterial load with the extent of vesical leukocyte infiltration, the number of F4/80$^+$ CD11c$^-$ MΦ, of Gr1$^+$ CD11c$^-$ F4/80$^-$ MHC class II$^-$ NΦG and of CD11c$^+$ DC in the bladder were determined by adding standardized numbers of Lx particles into the analyzing tubes (Fig. 2 and Table 2). This staining protocol for these different cell types was chosen to avoid inclusion of F4/80$^+$ CD11c$^+$ DC (41) into the MΦ count, and to prevent inclusion of Gr1$^+$ F4/80$^+$ monocytes (24) or CD11c$^+$ Gr1$^+$ MHC II$^+$ pDC (15) into the NΦG count. In non-infected animals, very few leukocytes were detectable. Already 2 hours after infection numbers of MΦ, NΦG and DC had increased significantly. The numbers of all leukocyte subsets peaked 14 hours after infection, whereas NΦG was the most abundant cell subset. Numbers of all leukocyte populations were reduced already after 3 days. Number of NΦG had decreased greatly after 3 days, whereas DC and MΦ remained elevated (Fig. 2 and Table 2).

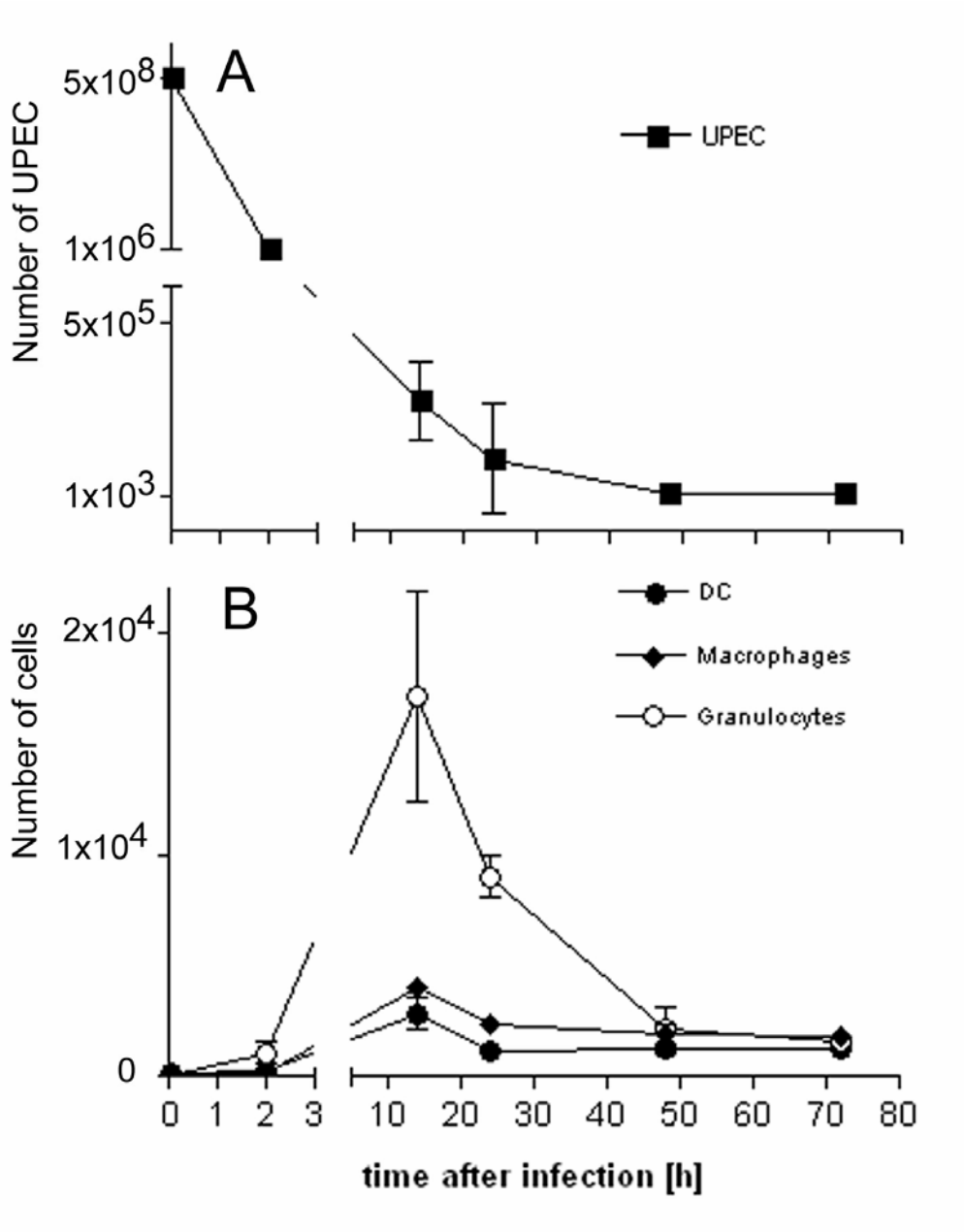


Fig. 2. Kinetics of bacterial clearance and leukocyte infiltration in UTI.

C57BL/6 mice were injected transurethrally with $5x10^8$ *E.coli* 536. At various time points, bladders were rinsed extensively with PBS *in situ* and removed for analysis. To determine the bacterial load, the bladders were mechanically homogenized. Aliquots were dispersed on CPS plates and incubated at 37°C. CFU of UPEC on the plates were counted after 14 hours. For the time point of infection, the number of instilled UPEC was given (A).

To determine the number of infiltrating leukocytes, bladders were digested with collagenase, filtered on 100 µm mesh, Fc receptors were blocked and cells were stained for flow cytometrical analysis. The numbers of $CD11c^+$ DC (●), $F4/80^+$ $CD11c^-$ MΦ (♦) and $Gr1^+$ $CD11c^-$ $F4/80^-$ MHC class II^- NΦG (○) were determined by adding standardized numbers of Lx particles to the analyzing tubes. Dead cells were excluded using Hoechst 33258 (B).

Data are representative for >5 individual experiments. Given are mean ± SD in groups of 5 mice.

Time after infection	DC	MΦ	NΦG
0 h	144 ± 36	109 ± 34	41 ± 26
2 h	323 ± 75	149 ± 27	1053 ± 700
14 h	2845 ± 976	4035 ± 586	17096 ± 6625
24 h	1199 ± 296	2408 ± 471	9001 ± 1310
48 h	1255 ± 144	1928 ± 453	2126 ± 1061
72 h	1311 ± 83	1822 ± 502	1622 ± 87

Table 2. Number of DC, MΦ and NΦG in the bladder

7.2. Infection of epithelial cells and recruitment of leukocytes into the bladder

The mechanism of adhesion and invasion of UPEC and the infiltration of mono- and polymorphonuclear leukocytes were further characterized by electron microscopy (EM) (Fig. 3). We detected adhesion (Fig. 3A) and invasion of instilled UPEC (Fig. 3B) into uroepithelial cells. Infected cells showed pod like structures on their surface demonstrating bacteria embedded in a fibrous matrix (Fig. 3B). Each bacterium was surrounded by an electron-lucent halo separating the bacteria from each other and disconnecting the bacteria from the epithelial cell content (Fig. 3B). Infection of uroepithelial cells also induce production of mucus to protect cells from infection (Fig. 3A and E). These data suggested that this bacterial factory is tightly packed into biofilm-like pod structures in uroepithelial cells, which enable persistence of this UPEC strain, as recently reported by others (3).

EM also revealed infiltrating mono- and polymorphnuclear leukocytes in the bladder, which were mostly infiltrating the epithelium (Fig. 3C and F). Intraepithelial NΦG form a barrier underneath infected uroepithelial cells and underwent apoptosis (Fig. 3A, B and D). We locally found close contact of apoptotic NΦG and infiltrating mononuclear cells in the submucosa, but not in the epithelium (Fig. 3D). These mononuclear leukocytes showed typical lysosomes, which is one characteristic feature of tissue MΦ. Furthermore, we found intraepithelial mononuclear leukocytes in the epithelium and bladder submucosa (Fig. 3F and G), which contained neither the segmented nuclei of NΦG, nor large lysosomes typical of MΦ. These cells showed abundant perinuclear organelles and protrusions devoid of organelles (Fig. 3E and G) and were compatible with those of DC in non-lymphoid tissue (40).

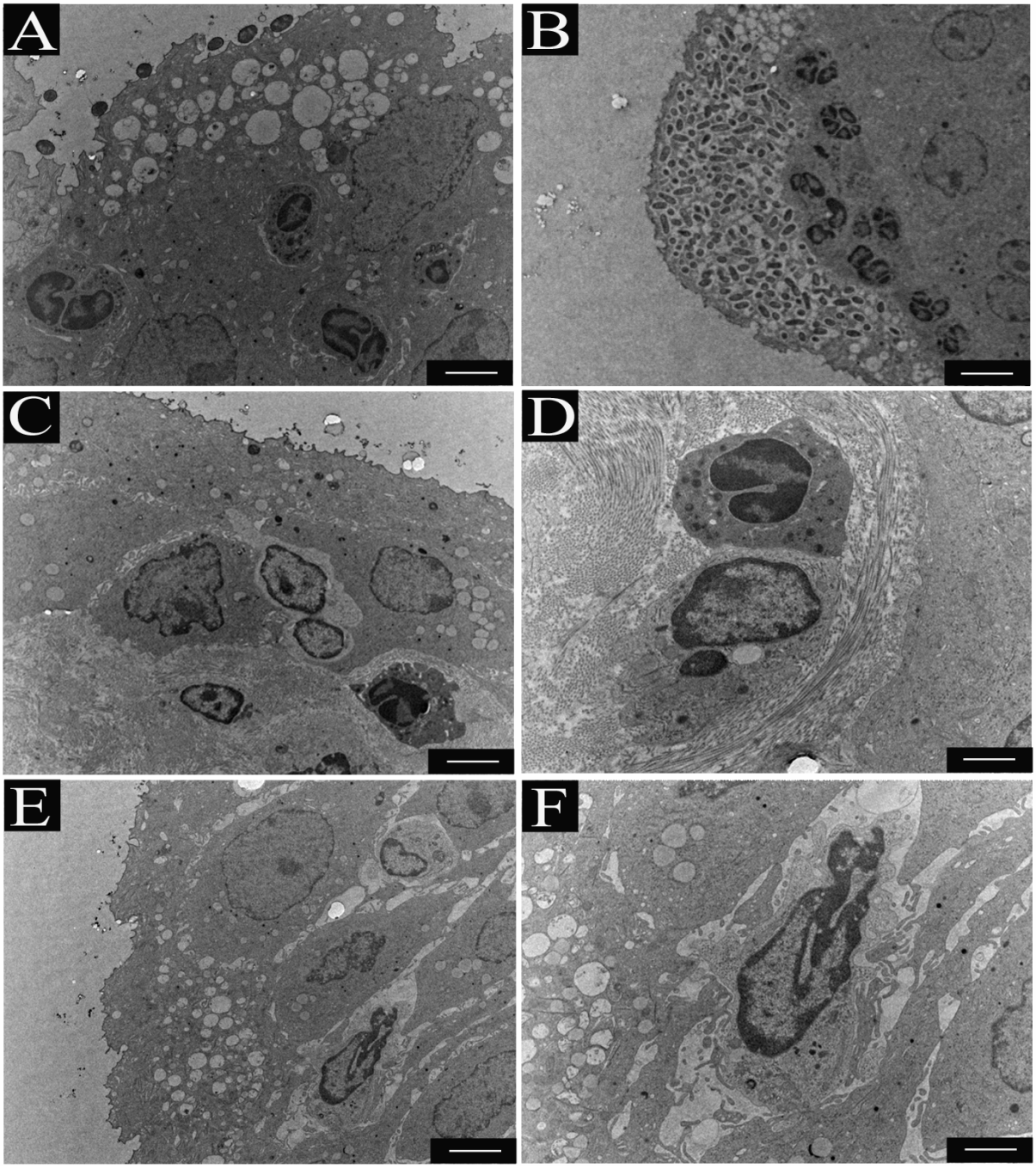


Fig. 3. EM of infected bladders.

C57BL/6 mice were infected transurethrally with $5x10^8$ *E.coli* 536. After 24 hours, sections of the bladder were analyzed by electron microscopy. The images are representative for more than 5 mice analyzed. The bars in the lower right corners indicate 20µm (A,C,E), 5µm (D,F) or 30µm (B).

7.3. DC are recruited into the epithelium after infection

This infiltrating cell subset, which showed typical characteristics of DC, was further analyzed in infected and non-infected bladders by immunohistochemistry (IHC) and immunofluorescence (IF) (Fig. 4). To this end, bladder sections were stained for the murine DC marker CD11c. This approach demonstrated $CD11c^+$ mononuclear leukocytes with dendritic morphology in the uroepithelium and bladder submucosa (Fig. 4B and C). These vesical leukocytes were absent from the bladders of non-infected mice (Fig. 4A). We also found that infiltrating DC were in close contact to instilled *E.coli* 536gfp and furthermore had taken up UPEC (Fig. 4D).

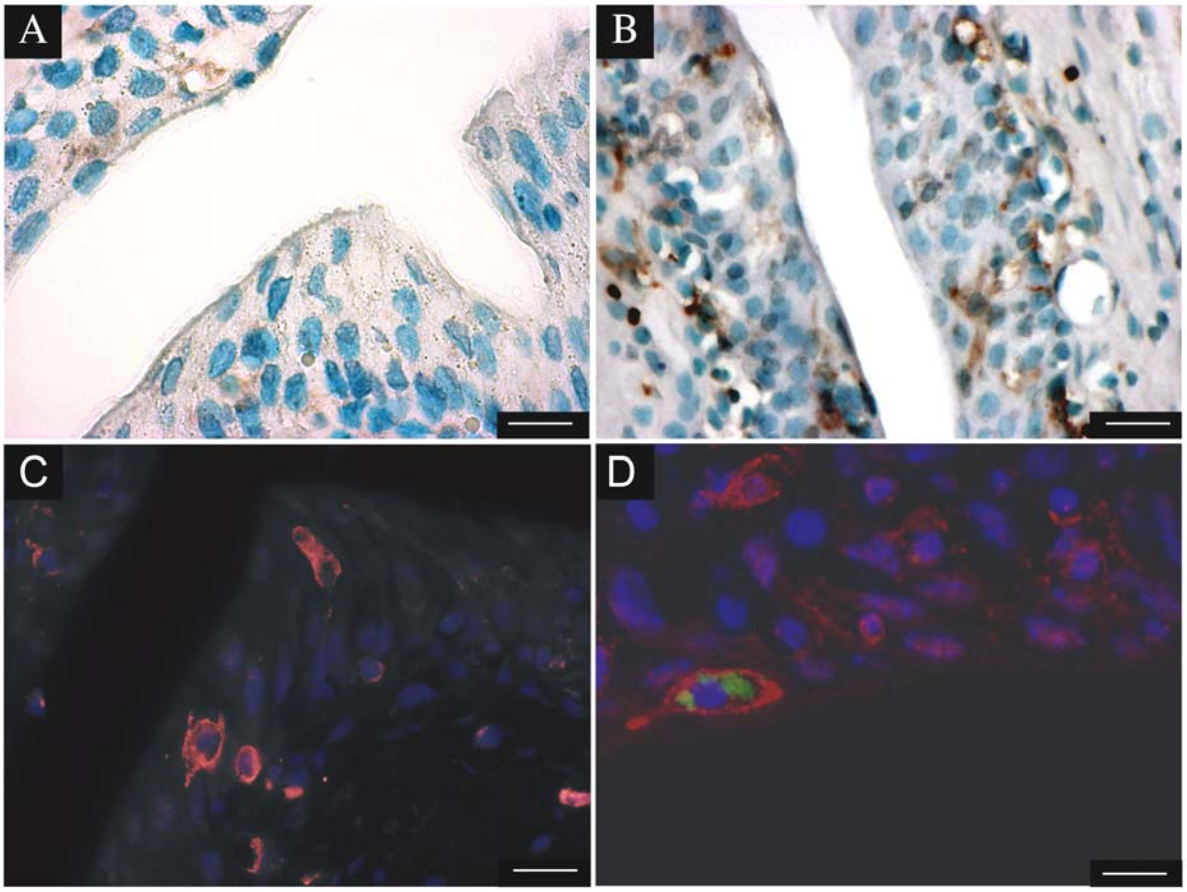


Fig. 4. Recruitment of DC to the bladder.

C57BL/6 mice were infected transurethrally with $5x10^8$ *E.coli* 536. After 24 hours, sections of the bladder were stained for CD11c and analyzed by immunohistochemistry (A, B) and immunofluorescence (C, D). $CD11c^+$ cells were brown (A, B) or red (C, D). Counterstaining was performed with methyl green (A, B) or Hoechst 33258 (C, D). Inoculated *E.coli* 536gfp were green (D). The images are representative for more than 10 mice analyzed. The bars in the lower right corners indicate 20µm.

7.3.1. Characterization of DC infiltrating the bladder

To further characterize the DC infiltrating the bladder, expression of activation and maturation markers was determined. The few vesical DC in non-infected control mice expressed low or intermediate levels of CD40, CD80, CD86 and MHC class II. All costimulatory molecules and MHC II were markedly upregulated 24 hours after infection (Fig. 5A), indicating DC maturation. For CD80 and MHC class II, a DC subpopulation devoid of surface expression of these molecules were evident in infected mice, which may represent immature DC recruited from the circulation. Neither in the spleen, nor in subcutaneous lymph nodes, activated DC were detectable after infection, indicating that UPEC had not spread systemically.

Next, the subtype of infiltrating DC was determined. The few DC in non-infected bladders expressed F4/80 and CD11b (Fig. 5B, Table 3). One day after infection, only some DC devoid of these markers were detectable, while most were of the $CD11b^{+}$ phenotype (Fig. 5B, Table 3). DC expressing CD8α, Gr1 or B220 were absent, indicating that neither pDC nor $CD8\alpha^{+}$ were recruited in UTI (Fig. 5B, Table 3). In addition to DC, $F4/80^{+}$ $CD11c^{-}$ MΦ and $Gr1^{+}$ $CD11c^{-}$ $F4/80^{-}$ NΦG were evident (Fig. 5B, Table 3).

Closer examination of the $CD11b^{+}$ DC from infected mice revealed DC that expressed intermediate (see area Fig. 5B, 2nd row, 1st column) levels of CD11b. These $CD11b^{INT}$ DC were rare in the bladders of non-infected mice (Fig. 5B).

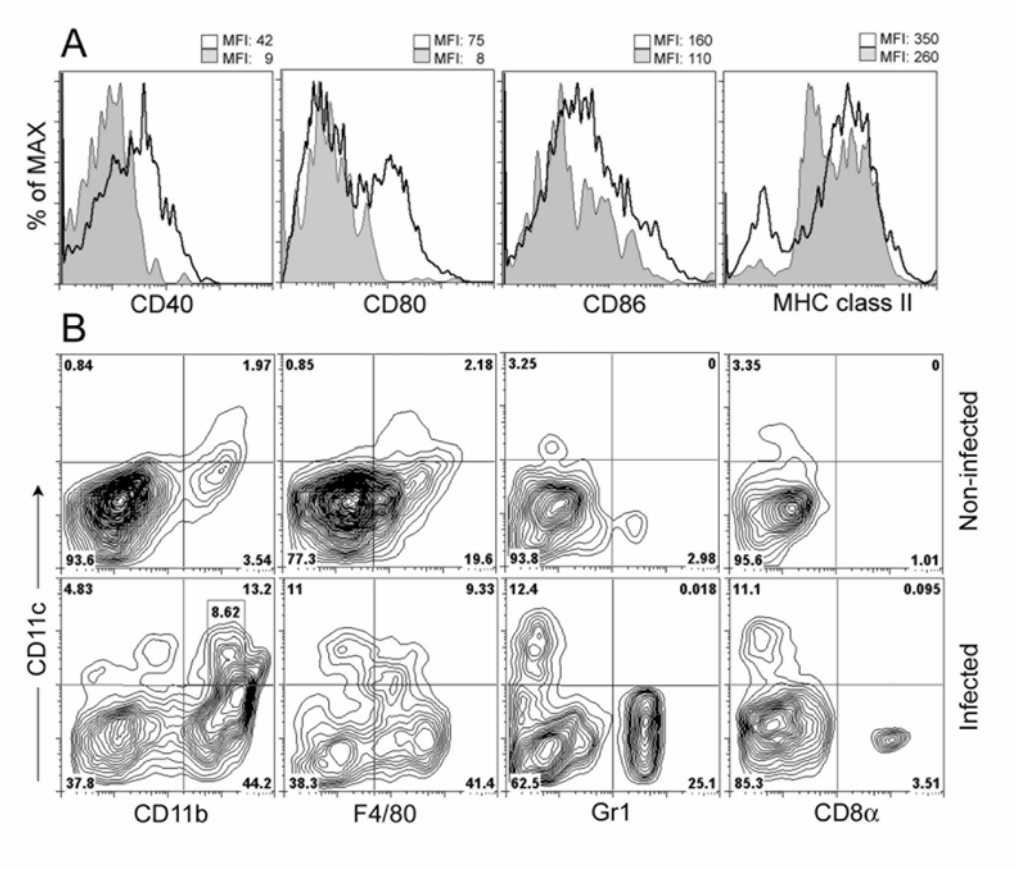


Fig. 5. Characterization of vesical DC in UTI.

C57BL/6 mice were infected with $5x10^8$ *E.coli* 536. After 24 hours, vesical DC were isolated and expression of surface molecules was determined.
Expression profiles in non-infected (grey area) versus infected mice (transparent area with thick line) were overlaid in histograms. Numbers indicate the mean fluorescence intensity (MFI) of these two cell populations (A).
Viable DC from infected and non-infected mice were stained for the DC subtype markers CD11b, F4/80, Gr1 and CD8α and analyzed by flow cytometry. Numbers indicate the cellular proportions in each quadrant. The area in the lower left dot-plot indicates $CD11b^{INT}$ DC (B).
Data are representative for >5 (A, B) individual experiments.

Time after infection	Non-infected	Infected
$CD11b^{LO/HI}$ DC	98 ± 13	821 ± 91
$CD11b^{INT}$ DC	0 ± 0	422 ± 21
$F4/80^+$ DC	61 ± 13	621 ± 78
$Gr1^+$ "plasmacytoid" DC	0 ± 0	2 ± 0.2
$CD8\alpha^+$ DC	0 ± 0	9 ± 1

Table 3. Numbers of different DC subtypes 24 hours after infection

C57BL/6 mice were infected with $5x10^8$ *E.coli* 536. After 24 hours, vesical DC were isolated by collagenase digestion and calculated by adding standardized numbers of beads to the samples.
Data are representative for >6 individual experiments. Given are mean ± SD in groups of 5 mice.

7.3.2. TNFα/iNOS-producing (Tip-) DC are recruited to the bladder in UTI

DC expressing intermediate levels of CD11b ($CD11b^{INT}$) and producing TNFα and iNOS (Tip-DC) have recently been shown to be critical in the innate defense against bacterial *Listeria monocytogenes* infection (77). We aimed to determine whether the vesical $CD11b^{INT}$ DC population identified in UTI (Fig. 5B) matched the criteria of Tip-DC and wether they exert different functionality than the conventional DC (cDC), which expressed high or low levels of CD11b ($CD11b^{LO/HI}$). Indeed, about 8-15 % of the vesical DC expressed iNOS, in contrast to less than 0.1% in non-infected bladders (Fig. 6). TNFα was produced by about 20-30 % of the vesical DC, in contrast to less than 0.1% in non-infected bladders (Fig. 6A). All $iNOS^+$ - and $TNF\alpha^+$ DC (Fig. 6B) expressed intermediate levels of CD11b, as described for Tip-DC (77). All cDC in UTI neither expressed TNFα nor iNOS.

Another characteristic of Tip-DC was their absence from CCR2-deficient mice and indeed, these mice lacked Tip-DC. (Fig. 6A). All DC in UTI expressed CCR2 (Fig. 6C), implying that Tip-DC and cDC must have been positive for this chemokine receptor. Finally, as opposed to Tip-DC and in contrast to cDC, these cells did not take up UPEC-derived antigens (Fig. 6D), consistent with the previous demonstration that Tip-DC were not involved in adaptive immune responses against bacterial pathogens (77).

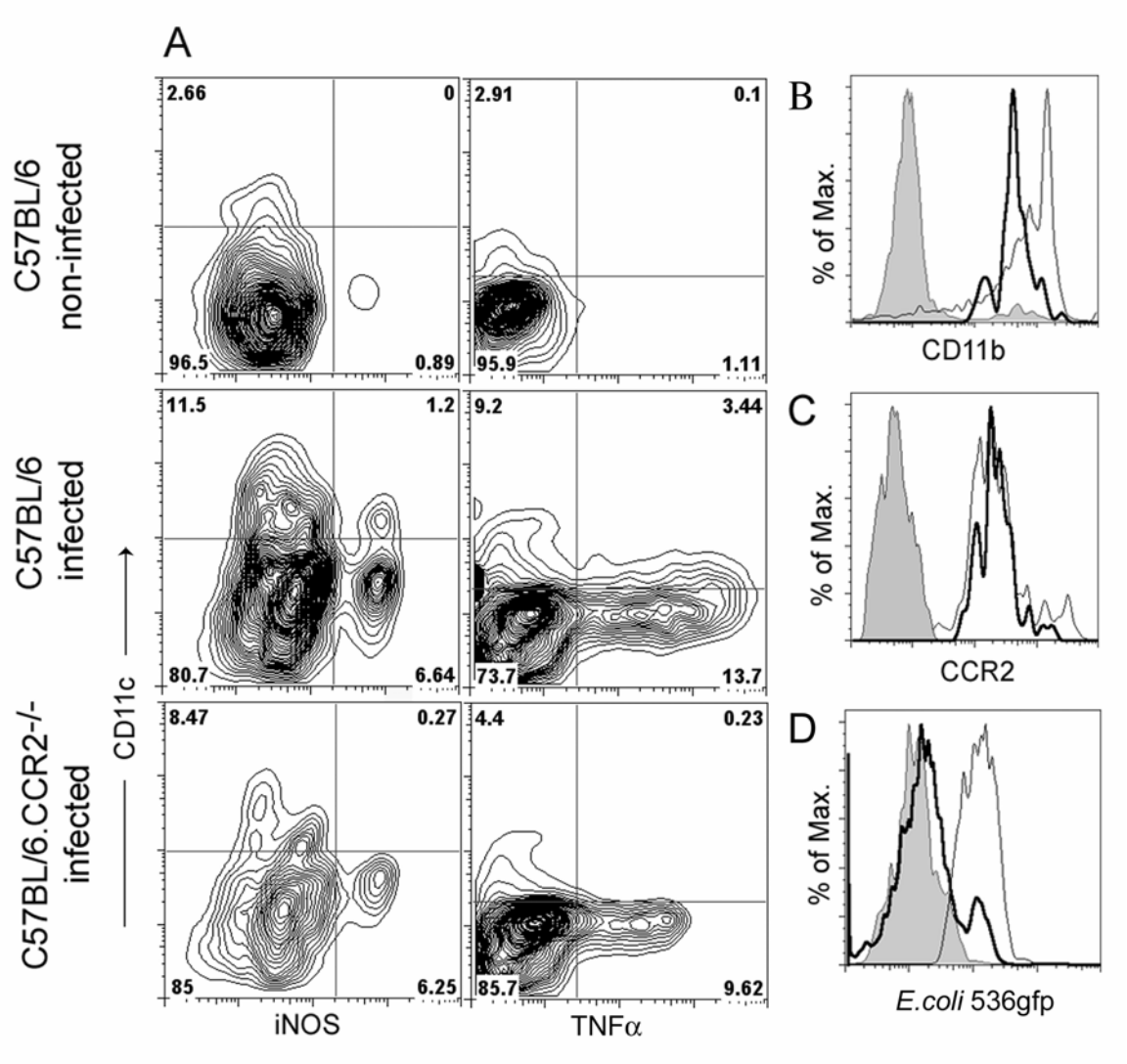


Fig. 6. TNFα/iNOS-producing (Tip-) DC are recruited to the bladder in UTI.

C57BL/6 or CCR2-deficient mice were infected transurethrally with $5x10^8$ *E.coli* 536 or *E.coli* 536gfp. After 24 hours, the bladders of infected mice and non-infected controls were digested with collagenase and analyzed for surface and intracellular molecules.
Cells isolated from infected and non-infected controls were stained for CD11c and for intracellular expression of iNOS and TNFα (A).
Vesical DC from infected mice were analyzed for CD11b (B) and CCR2 (C) expression. The thick line indicates expression by Tip-DC, the thin line that of cDC. Grey areas represent isotype controls.
C57BL/6 mice were infected with $5x10^8$ *E.coli* 536gfp. After 24 hours, vesical DC were isolated and fluorescence uptake was determined on Tip-DC (thick line) and cDC (thin line). The grey area shows background fluorescence after infection with non-fluorescent UPEC (D).

In summary, cDC and Tip-DC differed in phenotype and function. Tip-DC exerted innate immune functions like secretion of effector functions, whereas cDC were professional in taking up bacteria to induce adaptive immune responses (Table 4).

	TNFα and iNOS producing DC	Conventional DC
		
	Tip-DC	**cDC**
Gr1	–	–
CD11c	++	++
F4/80	+	+ or –
CD11b	INT	HI or LO
CD115	–	–
TNFα	+	–
iNOS	+	–
UPEC uptake	+	++
MHC class II	+	++
CD80	+	++

Table 4. Phenotype and function of cDC and Tip-DC.

7.3.3. Crucial role of CCR2 for the recruitment of MHC class II positive DC

In CCR2-deficient mice, DC numbers were found to be reduced to about 50% (Fig. 7A, B). This could not be explained by the absence of Tip-DC, as this population never constituted more than 15% of the vesical DC (Fig. 6A, C). Instead, this reduction was due to the selective absence of MHC class II^+ DC in CCR2-deficient mice (Fig. 7A, B). Interestingly, also MHC class II^+ non-DC were absent, consistent with previous findings that recruitment of MΦ to sites of infection depended on CCR2 (73) (50) (62). DC devoid of MHC II surface expression were not significantly altered in CCR2-deficient mice (Fig. 7A, B, C), indicating that immature DC use other receptors than CCR2 to migrate into infected bladders. Likewise, NΦG numbers were unchanged (Fig. 7C), consistent with previous reports showing recruitment of these cells by molecules other than CCR2.

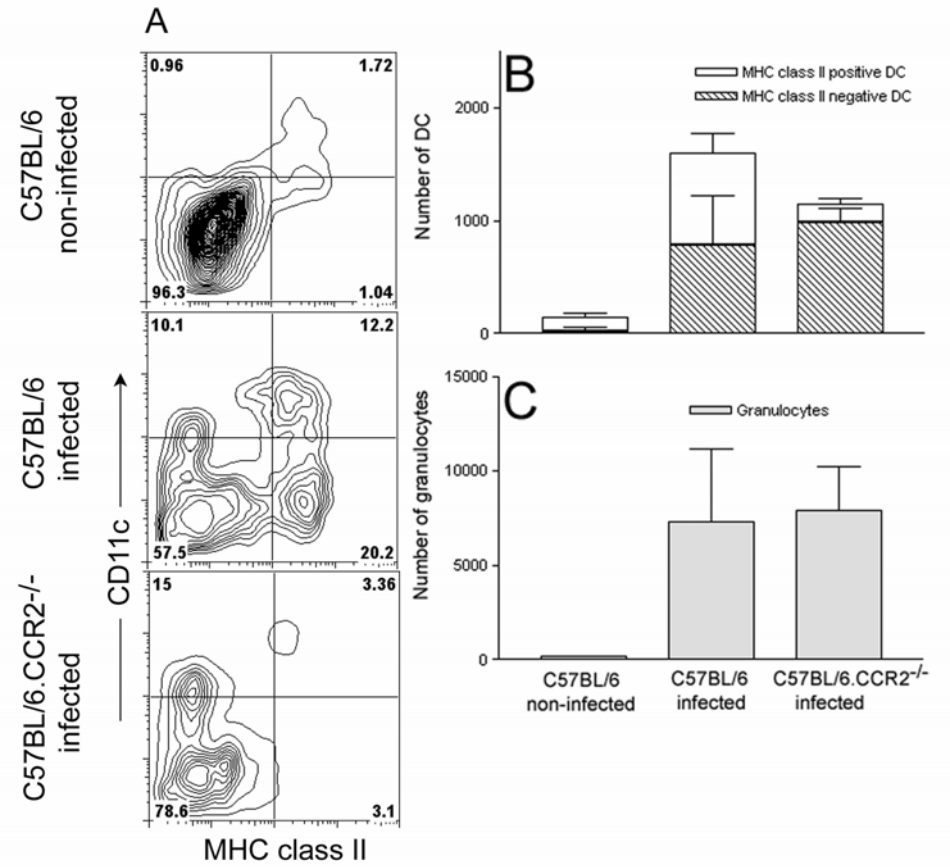


Fig. 7. CCR2 is required for the accumulation of mature DC in UTI.

C57BL/6 or CCR2-deficient mice were infected with $5x10^8$ *E.coli* 536. After 24 hours, vesical cells were analyzed for expression of MHC class II. Numbers indicate the cellular proportions in each quadrant. Shown in B and C are the mean ± SD of groups of 5 mice. Results are representative of three individual experiments.
The numbers of MHC class II^- DC (hatched bars) and those of MHC class II^+ DC (white bars) in single cell suspensions from the bladder were determined by flow cytometry. Both areas were stacked to yield the total numbers of DC (B).
In the same experiment, numbers of $Gr1^+$ $F4/80^-$ MHC class II^- NΦG were determined (C).

7.3.4. CCR2-dependent maturation after phagocytosis of UPEC

DC are important contributors in induction of adaptive immunity and in phagocytosis of antigen. As shown in Fig. 6D, cDC are able to phagocytose inoculated UPEC very efficiently and 40 to 66% of these DC were positive for instilled bacteria (Fig. 8). Neither DC in the spleen, nor in subcutaneous LN contained GFP-expressing UPEC or had upregulated activation markers, indicating that UPEC had not spread systemically. DC expressing MHC class II and CD80 contained UPEC (Fig. 8), indicating a correlation of phagocytosis of UPEC with these two molecules on DC. In CCR2-deficient mice, the number of these cells containing UPEC was reduced to about 30-35%, consistent with the reduced abundance of MHC class II^+ DC in the absence of this CCR (Fig. 8). The remaining DC presumably used molecules other than CCR2 for recruitment to the bladder. These data demonstrated that CCR2 contributed to the abundance of mature DC and phagocytosis of UPEC.

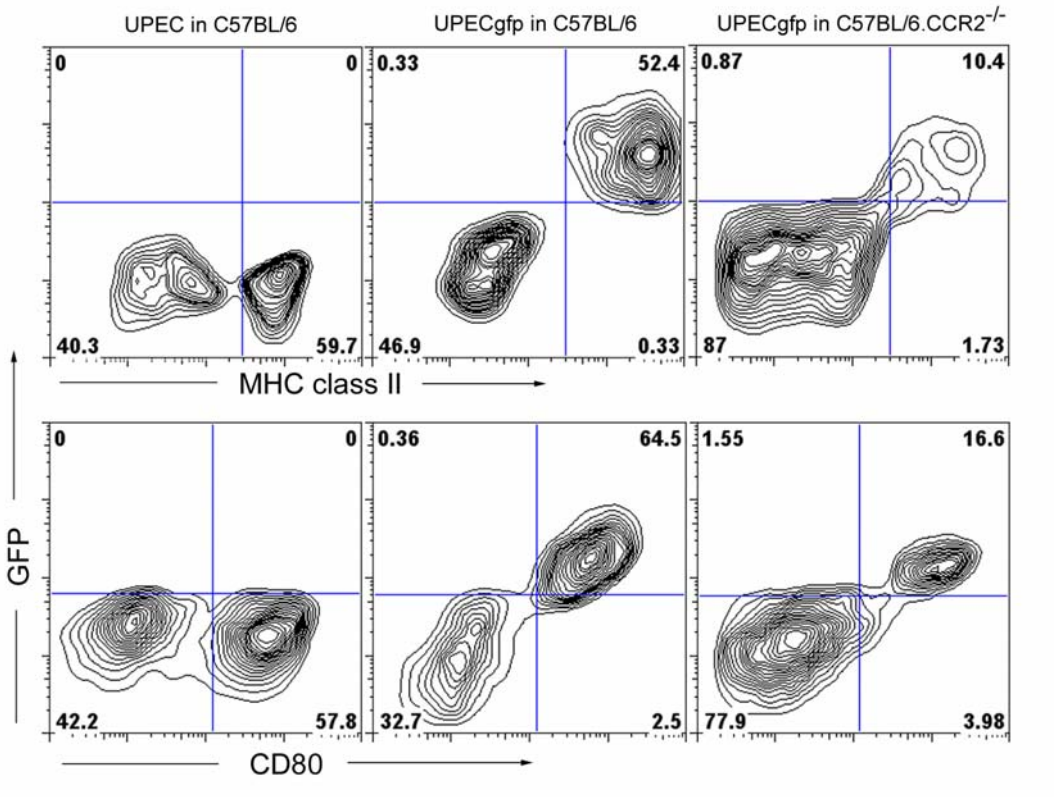


Fig. 8. DC expressing MHC II and costimulatory molecules phagocytose UPEC

C57BL/6 and CCR2-deficient mice were infected with $5x10^8$ *E.coli* 536 or *E.coli* 536gfp. After 14 hours, bladders were digested with collagenase, and viable CD11c-gated cells were analyzed for expression of MHC II, CD80 and for uptake of UPECgfp. Numbers indicate the cellular proportions in each quadrant. Results are representative for at least 3 experiments with 3 mice per group.

7.3.5. Neither DC, nor CCR2-dependent cells are required for clearance of UPEC

To investigate the requirement of Tip-DC for the defense against UTI, UPEC clearance from CCR2-deficient mice were determined. These mice cleared infection as efficiently as wild type controls (Fig. 9A), implying that Tip-DC were dispensable for bacterial clearance from the bladder in UTI.

To directly address the requirement of DC for the clearance of UPEC, CD11c-DTR mice that expressed the DTR under the influence of the CD11c promoter in DC were infected (39). Intraperitoneal injection of diptheria toxin resulted in depletion of more than 95 % of DC in the circulation and of about 90 % in the bladder (Fig. 9B). The number of NΦG was not diminished, at least not to a statistically significant extent (Fig. 9B). Bacterial clearance in DC-depleted CD11c-DTR mice was not different from that in non-depleted controls (Fig. 9A), indicating that DC were dispensable for innate clearance of instilled UPEC.

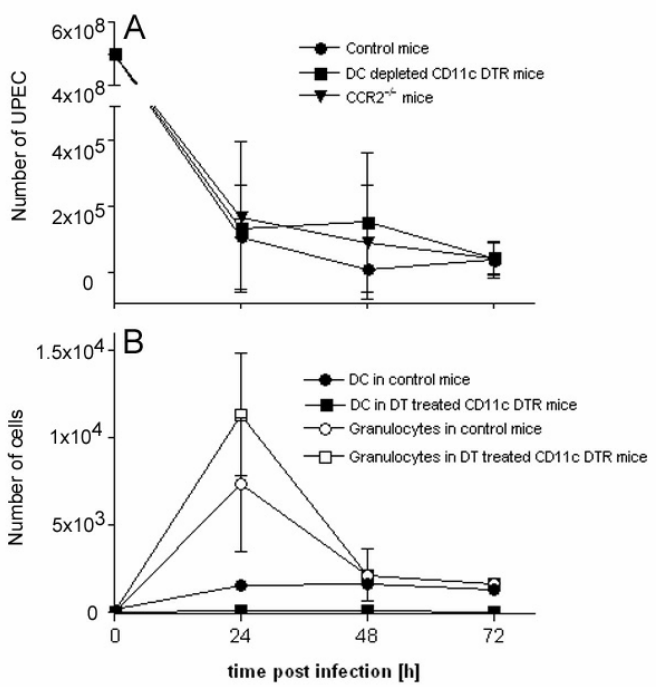


Fig. 9. Neither CD11c$^+$, nor other CCR2-dependent cells are required for clearance of UPEC in UTI.

CD11c-DTR mice (■), CCR2-deficient mice (▼) and C57BL/6 wild-type controls (●) were infected transurethrally with 5x10^8 *E.coli* 536. At various time points, numbers of CFU per bladder were determined. Bladder weight did not significantly differ 72 hours after infection (wildtype 20.4±1.8 mg, CCR2-deficient 21.2±1.7 mg) (A).
Numbers of CD11c$^+$ DC (■, ●) and those of Gr1$^+$ F4/80$^-$ MHC class II$^-$ NΦG (□, ○) in DT-treated CD11c-DTR/GFP mice (■, □) and in wild-type controls (●, ○) are given as mean ± SD of groups of 5 mice (B). Results are representative of four individual experiments.

7.3.6. CCR2-independent immune effector cells overlooked Tip-DC functionality

To elucidate the discrepancy between the requirement of Tip-DC in listeriosis and UTI, investigations were performed to determine whether other immune effectors may have substituted for the absence of Tip-DC. Indeed, the bladders of CCR2-deficient mice contained abundant TNFα– and iNOS producing $CD11c^{-}$ cells. These cells represented about 85 % of the total number of vesical $iNOS^{+}$ cells and 65 % of the total number of vesical $TNF\alpha^{+}$ cells in wild-type mice with UTI. These data reported above indicated that the loss of Tip-DC in CCR2-deficient animals caused a minor decrease (Fig. 10A and B) of both innate effector molecules. Given that iNOS-and TNFα producing non-DC expressed CD11b and Gr1, these cells represented NΦG and/or MΦ.

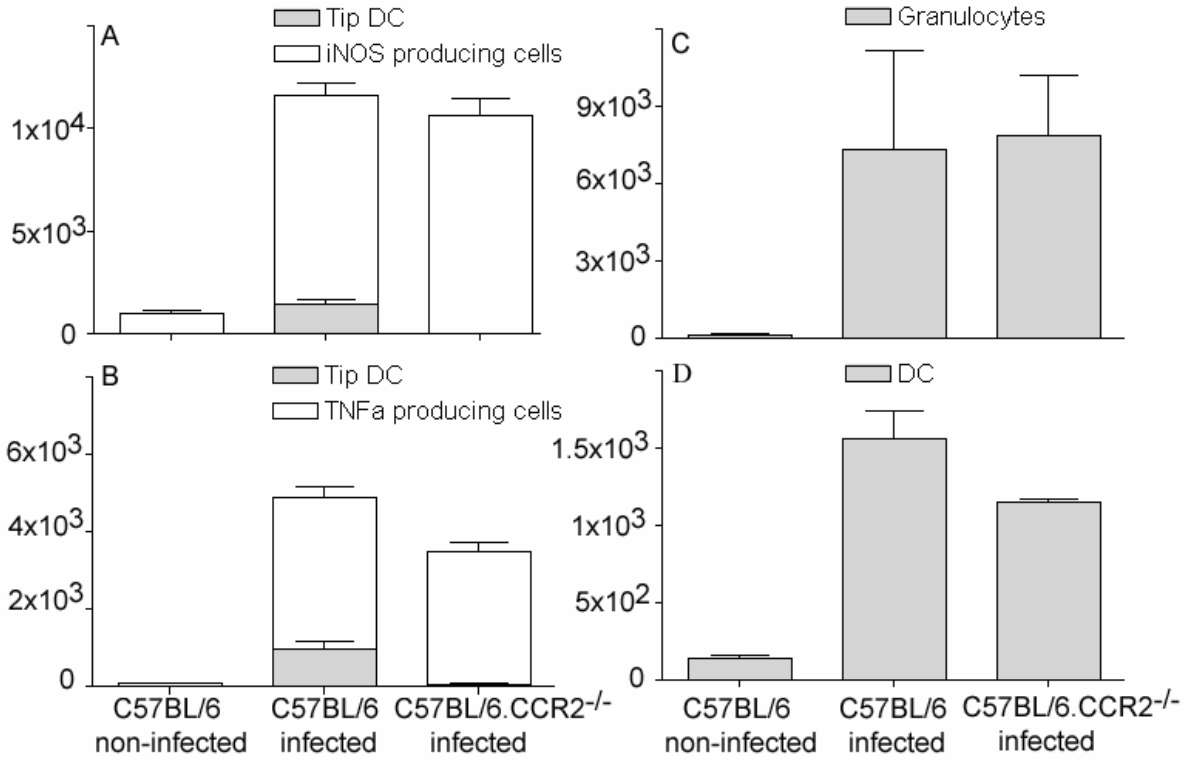


Fig. 10. CCR2-independent immune effector cells replace the functionality of Tip-DC in UTI.

C57BL/6 or CCR2-deficient mice were infected with $5x10^8$ *E.coli* 536. After 24 hours, the bladders of infected mice and non-infected controls were digested with collagenase and stained for surface molecules. Shown are the mean ± SD of groups of 5 mice. Results are representative of three individual experiments.
A and B. Numbers of $iNOS^{+}$ (A) or $TNF\alpha^{+}$ (B) DC (grey bars) and those of iNOS+ (A) or TNFα+ (B) non-DC (white bars) in single cell suspensions from the bladder were determined by flow cytometry. Bars were stacked to yield the total number of iNOS+ (A) or TNFα+ cells (B).
C and D.Numbers of NΦG (C) and of DC (D) in single cell suspensions from the bladder were determined by flow cytometry.

Next, as NO is an important effector molecule against UPEC (48) (77) (64), the cellular source of iNOS in infected bladders was determined (Fig. 11). To differentiate between MΦ and NΦG, the expression levels of F4/80 and Gr1 on $CD11c^{-}$ $iNOS^{+}$ cells were analyzed. NΦG express very high levels of Gr1, but were devoid of F4/80 expression. In contrast, F4/80 expression and less levels of Gr1 than NΦG on CD11c negative cells characterized MΦ.

As expected all iNOS producing cells were F4/80 positive, but expressed only low levels of Gr1 demonstrating that infiltrating MΦ, but not NΦG were the major source of this innate effector molecule.

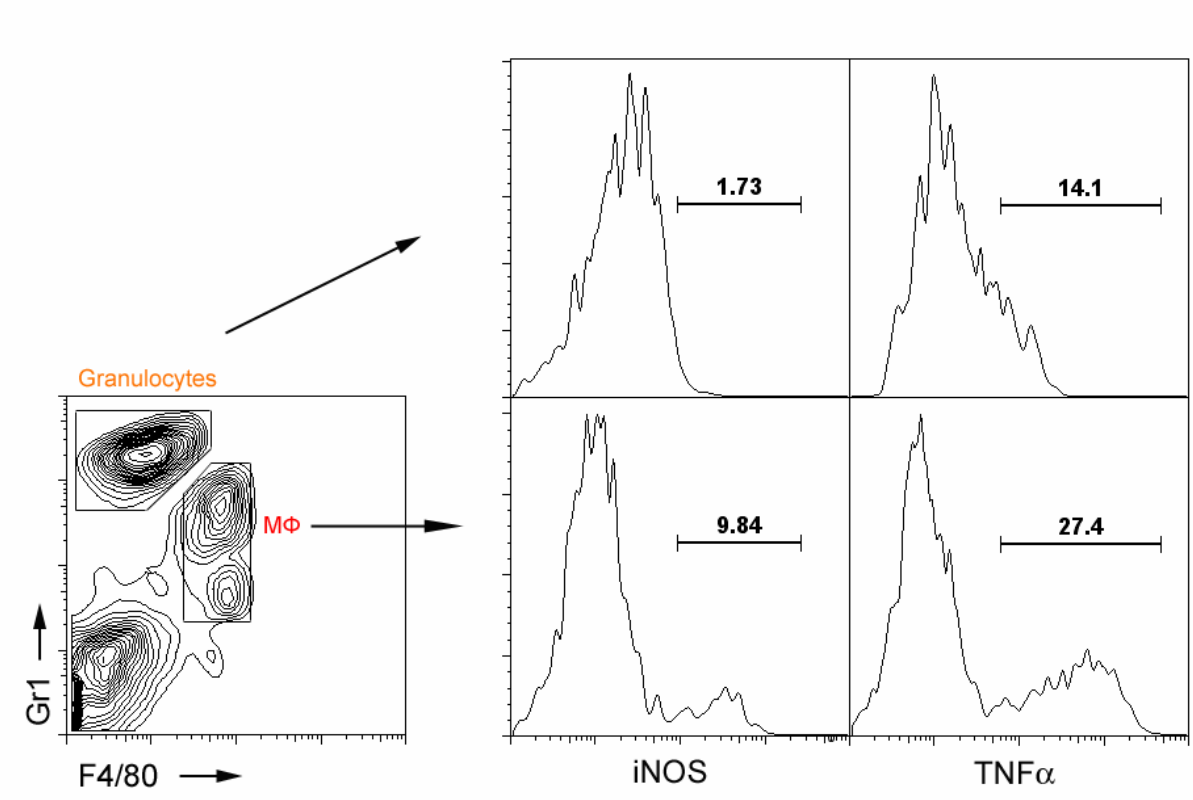


Fig. 11 . Mainly MΦ express iNOS and TNFα after infection with UPEC

C57BL/6 mice were infected with $5x10^{8}$ *E.coli* 536. After 16 hours, the bladders of infected mice were digested with collagenase and stained for F4/80, Gr1 and iNOS without restimulation. Results are representative of three individual experiments.

7.4. Heterogeneity of MΦ in different compartments involved in UTI

The data above indicated that MΦ are recruited and involved in the immune defense against UTI (Fig. 2, Fig. 11). To further study these immune cells, the composition of murine monocytes in the BM, the blood and in the bladder was investigated. Circulating monocytes in the blood expressed the surface molecules CD115 (M-CSF Receptor) and F4/80 (EGF-Receptor) and it has been shown previously (84) (24), that two blood monocyte subsets could be distinguished by expression of different levels of Gr1 (85) (Fig. 12). As NΦG or DC generally did not express CD115, analysis of cells expressing this molecule specifically targeted monocytes.

Monocytes in the circulation bearing low expression of Gr1 ($Gr1^{LO}$) have been associated with progenitors that constitutively enter non-inflamed tissue (24). In contrast, high expression levels of Gr1 ($Gr1^{HI}$) has been proposed to identify inflammatory monocytes that infiltrate infected and inflamed organs (76). $Gr1^{LO}$ monocytes have been shown to express high levels of CX_3CR1, but low to absent levels of CCR2. Conversely, inflammatory monocytes express low levels of CX_3CR1, but high levels of CCR2. It has been proposed that these chemokine receptors are responsible for the distinct migratory behavior of these subsets, but stringent proof of this hypothesis is lacking.

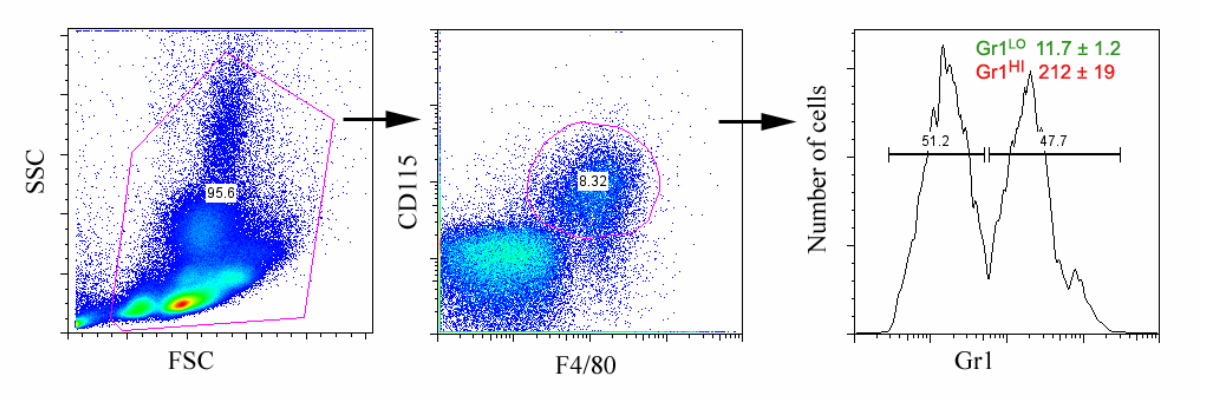


Fig. 12. Monocytes in the circulation of non-infected C57BL/6 mice

Whole blood was subjected to red cell lysis using RCB buffer, washed twice in DMEM and expression levels of CD115, F4/80 and Gr1 were analyzed. Results are representative of at least ten individual experiments.

7.4.1. MΦ subsets in organs involved in UTI

To investigate whether these two monocyte subsets were also present in the UTI model, the composition of monocytes in organs involved in this infection were analyzed (Fig. 13). To this end, expression levels of Gr1 on $F4/80^+$ and $CD115^+$ cells were analyzed in the BM, the blood and the bladder and the proportions of monocytes (BM and blood) and MΦ (bladder) were determined. In addition, absolute cell numbers of MΦ in the bladder were analyzed. In the BM more than 90% of CD115 and F4/80 positive monocytes in non-infected and infected mice were of the $Gr1^{HI}$ subset (Fig. 13A and B). In contrast, a higher proportion of $Gr1^{LO}$ monocytes were found in the blood of non-infected and infected mice, but significant differences in the ratio of $Gr1^{LO}$ and $Gr1^{HI}$ monocytes in the BM and the blood of infected and non-infected controls were not observed (Fig. 13B). These results suggested that local infection of mice with UPEC did not lead to systemic alterations of monocyte subsets, in contrast to chronic systemic inflammatory conditions such as atherosclerosis (F. Tacke, personal communication) or listeriosis (76).

Like in the BM and the blood, MΦ in non-infected and infected bladders were comprised of $Gr1^{LO}$ and $Gr1^{HI}$ cells (Fig. 13A and B). In contrast to the blood and the BM, the proportion of $Gr1^{HI}$ MΦ in the infected bladder was increased significantly (Fig. 13B, C). Furthermore, the absolute numbers of $Gr1^{LO}$ and $Gr1^{HI}$ monocytes in infected bladder were increased over the non-infected bladder (Fig. 13C), indicating that both monocyte subsets are recruited in UTI.

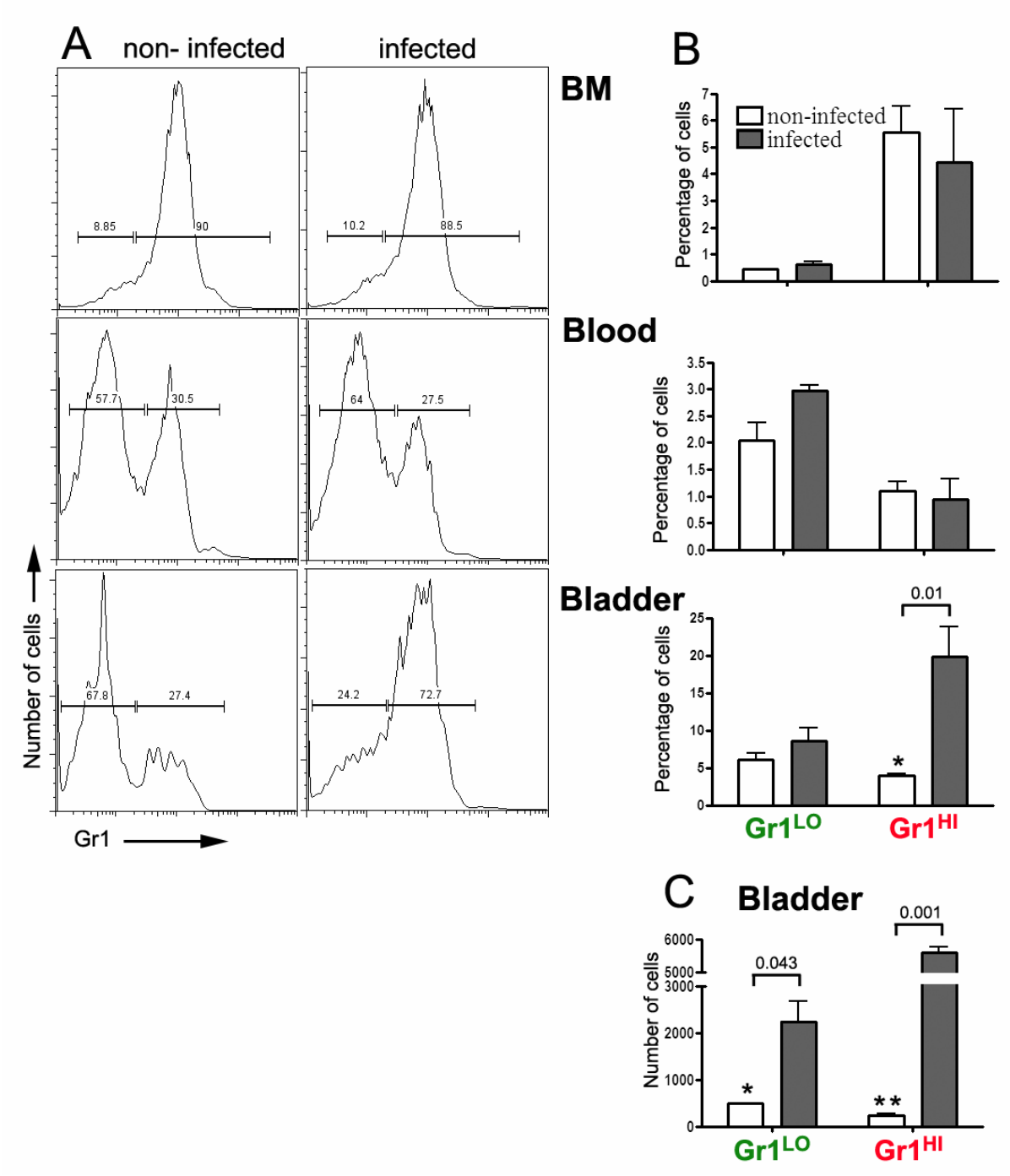


Fig. 13. Increased abundance of Gr1^{LO} and Gr1^{HI} MΦ in infected bladders

C57BL/6 mice were infected with *E.coli* 536. After 14 hours, infected and non-infected control mice were sacrificed and cells from the BM, the blood and the bladder were isolated. Viable, F4/80 and CD115 gated cells were analyzed and Gr1 positive cells were divided into Gr1^{LO} and Gr1^{HI} monocytes. For the BM, the blood and the bladder the ratio between all viable cells and the monocyte subsets were analyzed (A, B).
Additionally absolute cell numbers in the bladder of both monocyte subsets was calculated in non-infected and infected bladders (C).
Data are representative of experiments repeated three times with three mice per group. One asterisk indicates a p value <0.05, ** p<0.01, *** p<0.001.

Others have shown different expression levels of CCR2 and CX_3CR1 on circulating $Gr1^{LO}$ and $Gr1^{HI}$ blood monocytes (21, 24). To investigate whether these findings held true also for vesical MΦ in UTI, the expression levels of the chemokine receptors CCR2 and CX_3CR1 were determined under homeostatic and infectious conditions (Fig. 14). Indeed, $Gr1^{LO}$ MΦ in the bladders expressed high levels of CX_3CR1^{HI}, whereas the $Gr1^{HI}$ MΦ subset expressed high levels of CCR2 (Fig. 14). These results indicated higher expression of CX_3CR1 on $Gr1^{LO}$ MΦ and increased levels of CCR2 on $Gr1^{HI}$ MΦ in UTI, consistent with observations by others in different infection models (76).

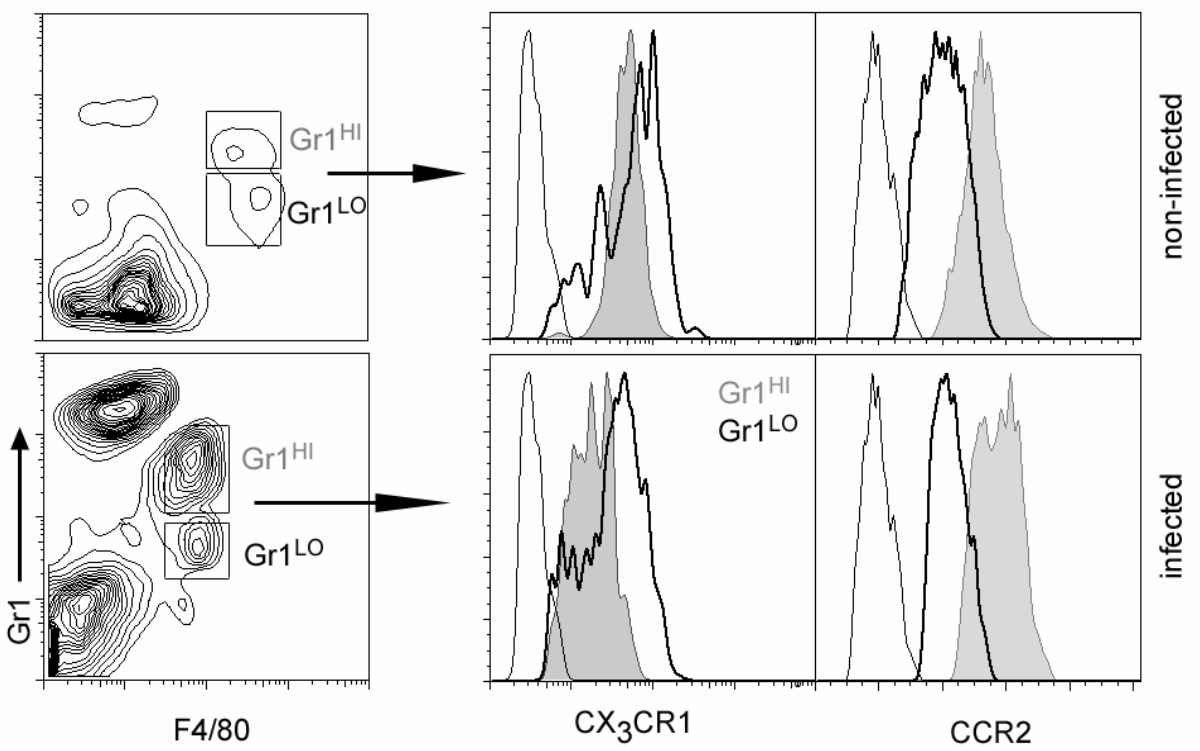


Fig. 14 . Expression of CX_3CR1 and CCR2 on bladder MΦ

Heterozygous CX_3CR1 mice were infected with $5x10^8$ *E.coli* 536. After 16 hours, the bladders of infected and non-infected controls were digested with collagenase and stained for CD115, F4/80, Gr1 and CCR2. CX_3CR1 and CCR2 expression on CD115 and F4/80 positive cells were analyzed. The weak black line represents the isotype control for the CCR2 staining and the background fluorescence in C57BL/6 mice. Shown are the mean ± SD of groups of 3 mice. Results are representative of three individual experiments.

7.4.2. Function of MΦ in UTI

An unresolved question concerns the functionality of the $Gr1^{LO}$ and $Gr1^{HI}$ MΦ in infections. To this end, the effector functions of these immune cell subsets in UTI were studied. The expression levels of the antimicrobial effector molecules iNOS and ß-defensins 1, the ability to phagocytose inoculated UPEC and TNFα secretion were analyzed in MΦ from infected animals and compared to infiltrating NΦG (Fig. 15). About 40% of the $Gr1^{LO}$ MΦ and 65% of the $Gr1^{HI}$ MΦ subset in the infected bladder expressed ß-defensin 1 with similiar expression levels. Strikingly, iNOS expression was confined to $Gr1^{LO}$ MΦ, while $Gr1^{HI}$ MΦ and NΦG were completely devoid of this molecule. Phagocytosis of UPEC could be demonstrated for MΦ and NΦG, but the percentage of cells that had phagocytosed UPEC were significantly higher in NΦG than in MΦ. In contrast, the mean fluorescence intensity (MFI) of TNFα–secreting cells was significantly higher in MΦ than in NΦG. Furthermore the percentage of TNFα producing $Gr1^{HI}$ MΦ was significantly higher than of $Gr1^{LO}$ MΦ. These results demonstrated that each cell subset possessed a characteristic pattern of effector functions against UPEC.

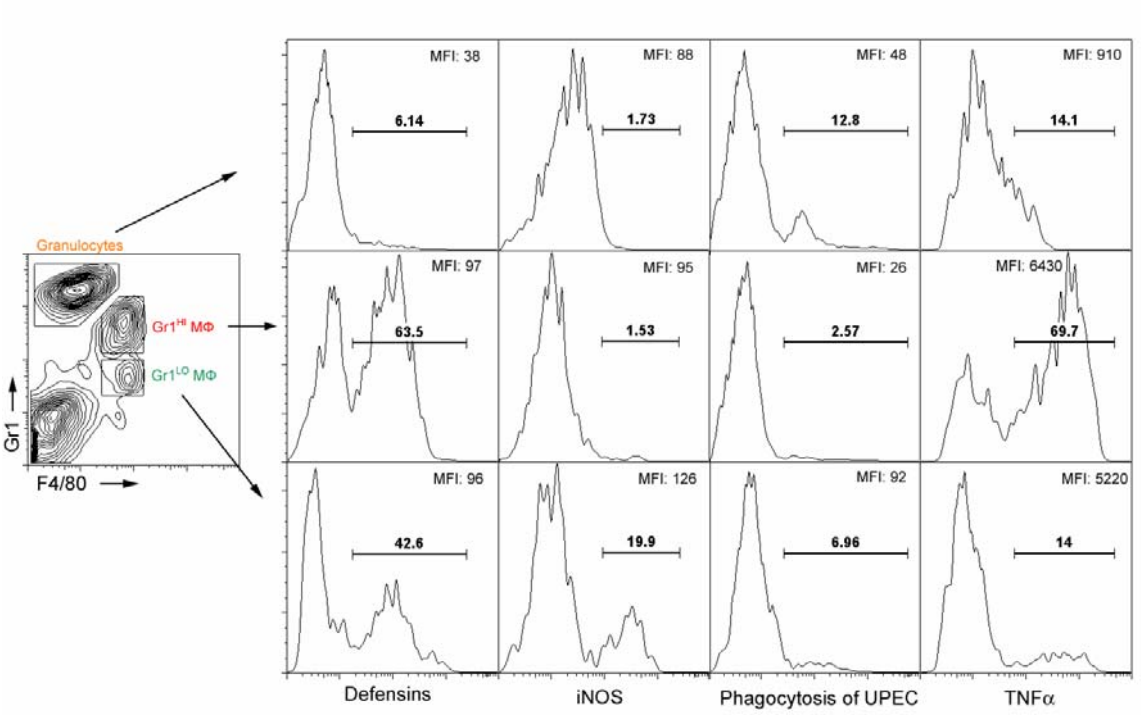


Fig. 15. Function of MΦ and NΦG after exposure to UPEC

C57BL/6 mice were infected with $5x10^8$ *E.coli* 536 or *E.coli* 536gfp to analyze phagocytic activity. After 16 hours, the bladders of infected mice were digested with collagenase and stained for F4/80, Gr1, β-defensin 1, iNOS and TNFα. The mean fluorescence intensity (MFI) represented the gated cells in the histogram presented. Results are representative of three individual experiments.

To demonstrate the contribution of each cell subset to the absolute amount of these antimicrobial functions described above, the portion per cell subset was determined (Fig. 16). $Gr1^{LO}$ MΦ were the principal cell subset expressing iNOS, whereas $Gr1^{HI}$ MΦ were the major source of TNFα in the infected bladder. Inoculated bacteria were predominantly taken up by the most abundant NΦG. The murine β-defensin-1 has been shown to be mostly expressed by epithelial cells, but in UTI this defensin was widely expressed on several cell subsets including phagocytes examined. This calculation suggested again that each immune cell subset possessed a characteristic effector function against UTI.

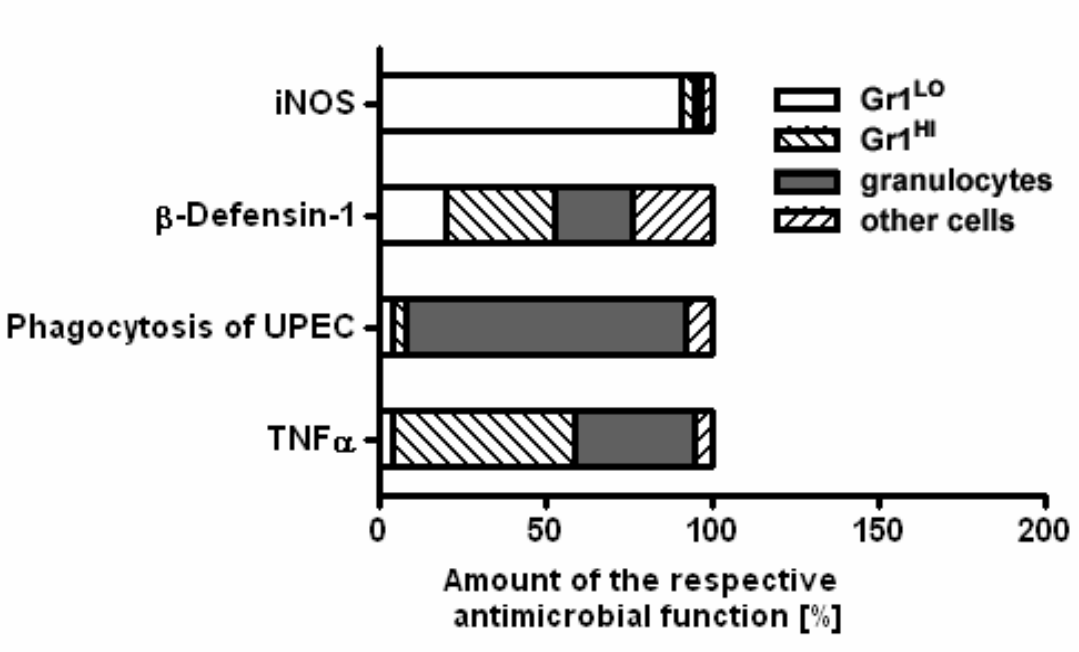


Fig. 16. Portion of antimicrobial functionality in the infected bladder

C57BL/6 mice were infected with $5x10^8$ *E.coli* 536 or *E.coli* 536gfp to analyze phagocytic activity. After 16 hours, the bladders of infected mice were digested with collagenase and stained for F4/80, Gr1, β-defensins 1, iNOS and TNFα. Results are representative of three individual experiments.

7.4.3. Table 5. Summary and comparison of different phagocytes involved in UTI

	NΦG	$Gr1^{LO}$ MΦ	$Gr1^{HI}$ MΦ
			
Gr1	+++	+	++
CD11c	–	+	–
F4/80	–	+	+
CD11b	++	++	++
CD115	–	+	+
CCR2	–	–	+
CX_3CR1	–	++	+
iNOS	–	+	–
β-defensin 1	+	+	+
UPEC uptake	+++	+	+
TNFα	+	++	–

7.5. Tracking of monocytes after *in vivo* labeling

To investigate whether the vesical $Gr1^{LO}$ and $Gr1^{HI}$ MΦ populations in UTI were derived from circulating $Gr1^{LO}$ and $Gr1^{HI}$ monocytes, a recently described labeling technique using fluorescent Lx beads was employed (84). As shown previously, circulating $Gr1^{LO}$ blood monocytes could be labeled by injection of fluorescent Lx beads into the tail vein (84), which were then phagocytosed predominantly by this subset. However, labeling of $Gr1^{HI}$ monocytes required introduction of Lx beads in the absence of circulating $Gr1^{LO}$ monocytes. This was achieved by depleting monocytes with clodronate-liposomes (clo-lip) as described previously (84). The $CCR2^{+}$ $Gr1^{HI}$ monocyte subset reconstituted the circulation already after 24 hours, whereas CX_3CR1^{+} $Gr1^{LO}$ remained absent for several days (84). In the absence of $Gr1^{LO}$ monocytes, injected Lx beads were taken up by $Gr1^{HI}$ monocytes (84), which received the beads in the BM from other cells, particularly from marginal zone B cells and NΦG that carried the particles to the BM. This labeling system allowed selective labeling of either circulating $Gr1^{LO}$ or $Gr1^{HI}$ monocytes.

To demonstrate efficient depletion of monocytes after administration of clo-lip into the tail vein and reconstitution of the blood with $Gr1^{HI}$ monocytes, mice were sacrificed 3 days after introducing the clo-lip into the tail vein and the ratio of $Gr1^{LO}$ and $Gr1^{HI}$ monocytes were determined (Fig. 17). Three days after depletion, 90% of all circulating monocytes expressed high levels of Gr1, as the blood was reconstituted by $Gr1^{HI}$ monocytes from the BM. By adoptive transfer experiments these $Gr1^{HI}$ monocytes have been shown to give rise to $Gr1^{LO}$ monocytes within 3-6 days (84) (F. Tacke, personal communication). These findings demonstrated that the system to label $Gr1^{HI}$ monocytes worked as described by others (84)

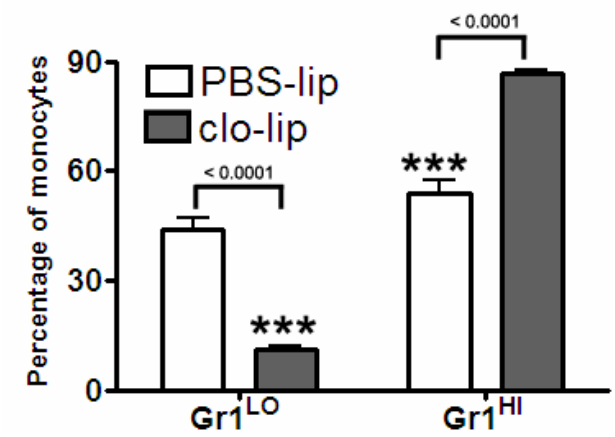
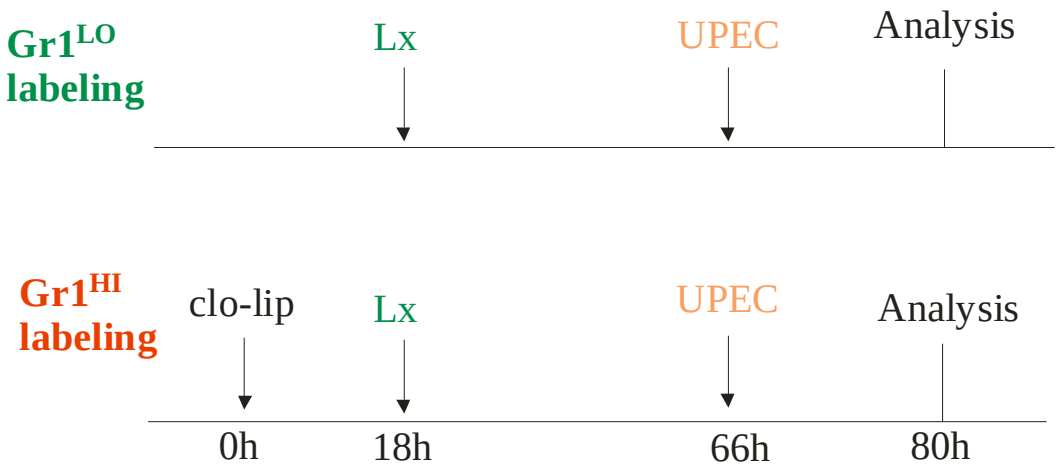


Fig. 17. Depletion efficacy of monocytes in infected C57BL/6 mice

Clo-lip were administered into the tail vein of C57BL/6 mice. 80 hours later, whole blood was subjected to red cell lysis using RCB buffer and washed twice in DMEM. Expression level of Gr1 on $CD115^+$ $F4/80^+$ gated monocytes in monocyte depleted (clo-lip) and non depleted mice (PBS-lip) were analyzed by flow cytometry. Shown are the mean ± SD of groups of 3 mice. Results are representative of three individual experiments. One asterisk indicates a p value <0.05, ** p<0.01, *** p<0.001.

To investigate the migration of Lx positive monocytes into the non-infected and into the infected bladder, either $Gr1^{LO}$ or $Gr1^{HI}$ monocytes were labeled by injection of the fluorescently labeled Lx beads. Thereafter, mice were infected with UPEC and 24 hours later infected and non-infected control mice were sacrificed (Scheme 3).



Scheme 3. Labeling of circulating $Gr1^{LO}$ and $Gr1^{HI}$ monocytes

To show that circulating monocytes had taken up injected Lx beads, blood cells were isolated from infected and non-infected controls and the percentage of Lx positive CD115 and F4/80 positive cells were analyzed (Fig. 18). Lx labeled $Gr1^{LO}$ or $Gr1^{HI}$ monocytes were present in the circulation from infected and non-infected controls. Furthermore, most of the cells had taken up one Lx beads, whereas only some had endocytosed multiple Lx beads (Fig. 18). Approximately 10 to 15% of $Gr1^{LO}$ monocytes were labeled in infected and non-infected controls, whereas 35-50% of the $Gr1^{HI}$ subset was efficiently labeled in non-infected and infected mice. These data indicated that both monocyte subsets could specifically be labeled by this technique. Furthermore, the labeling of $Gr1^{HI}$ blood monocytes was more efficient.

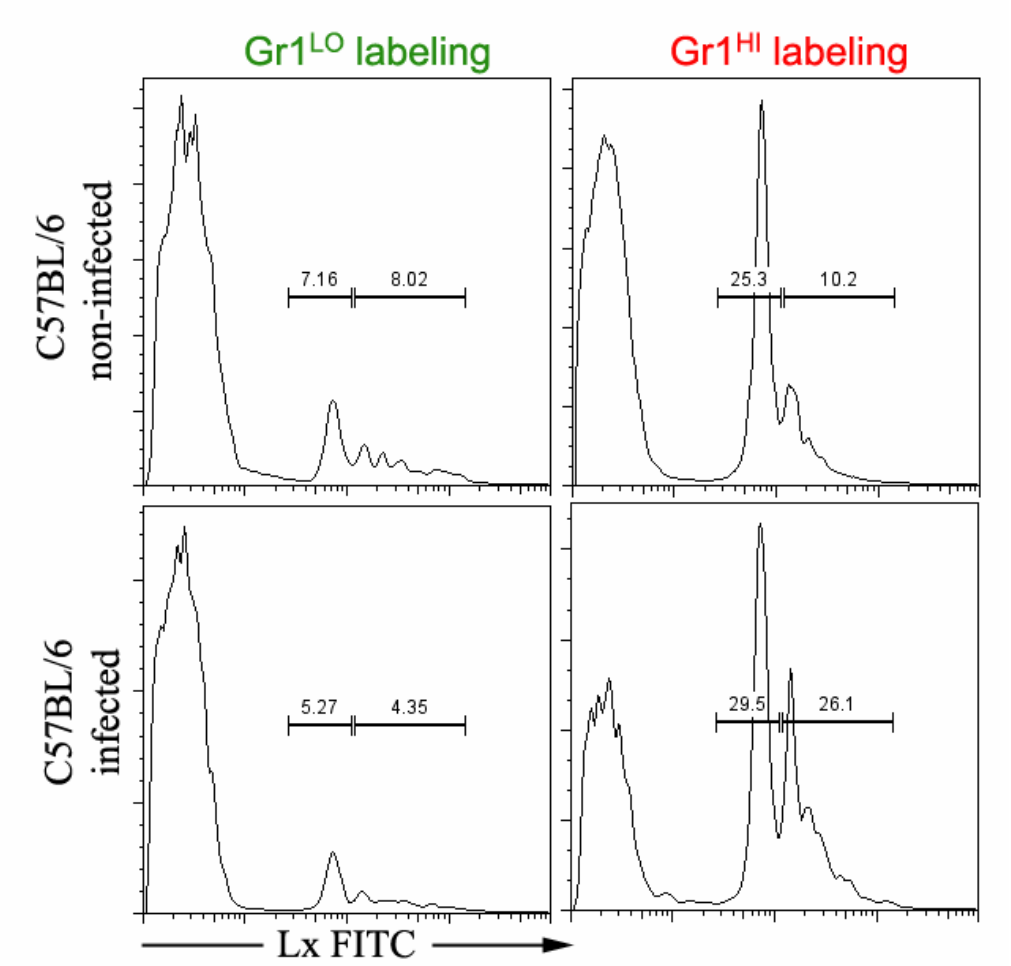


Fig. 18. Labeling of $Gr1^{LO}$ and $Gr1^{HI}$ monocytes in the circulation

Lx beads were administered into C57BL/6 mice to either label $Gr1^{LO}$ or $Gr1^{HI}$ monocytes. Whole blood was subjected to red cell lysis using RCB buffer and washed twice in DMEM and the percentage of single- or multi-bead positive $CD115^{+}$ $F4/80^{+}$ cells were analyzed. Shown are the mean ± SD of groups of 3 mice. Results are representative of three individual experiments.

7.5.1. Migration of monocytes into the infected and the non-infected bladder

The system established as described above was employed to study the migratory fate of both monocyte subsets into infected and non-infected bladders (Fig. 19A and B). To account for the different labeling efficiency of $Gr1^{LO}$ and $Gr1^{HI}$ monocytes with Lx beads, a normalization factor was used (Fig. 19B). This was possible because it has recently been shown that unlabelled and labeled circulating monocytes showed similar functionality and migratory behavior (F. Tacke, personal communication) (22). Using this system, it was found that the migration of $Gr1^{LO}$ and $Gr1^{HI}$ monocytes from the blood into the bladder under inflammatory conditions was significantly increased. These experiments suggested that bladder MΦ under homeostatic and infectious conditions originated both from $Gr1^{HI}$ and $Gr1^{LO}$ blood precursors.

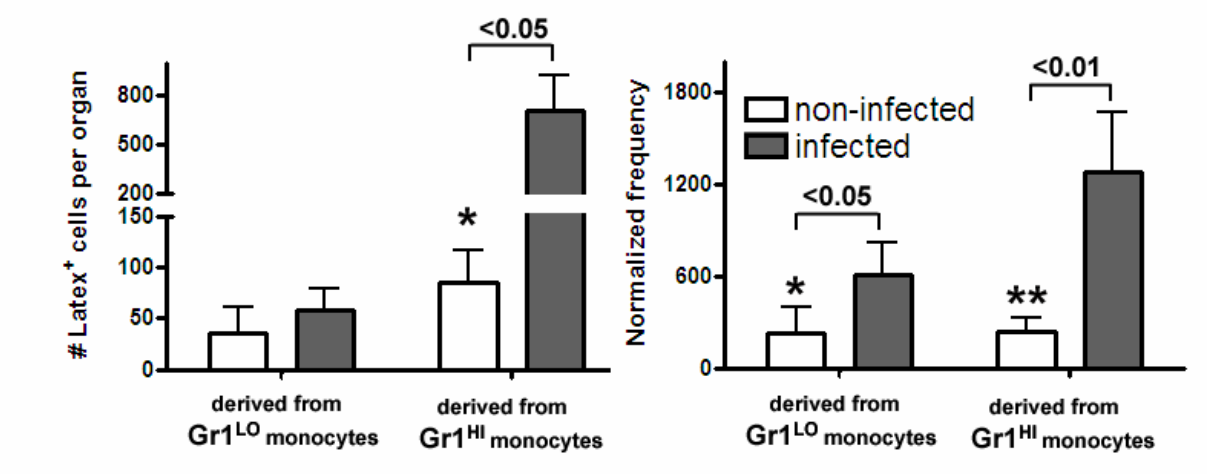


Fig. 19. Migration of monocyte subsets from the circulation into the bladder

Circulating $Gr1^{LO}$ or $Gr1^{HI}$ monocytes were labeled with fluorescent Lx beads. Two days later mice were infected with $5x10^8$ *E.coli* 536. After 24 hours, the bladders of infected and non-infected control mice were digested with collagenase and stained for CD115, F4/80 and Gr1. Lx expression on CD115 and F4/80 positive cells were analyzed. Shown are the mean ± SD of groups of 5 mice. Results were representative of three individual experiments. Asterisk indicate p values >0.05, where results were statistically significant. One asterisk indicates a p value <0.05, ** p<0.01, *** p<0.001.

7.5.2. Labeling of monocytes in the blood of CCR2-deficient mice

To determine the role of CCR2 in migration of monocytes into the bladder, CCR2-deficient mice were subjected to the labeling system. It has been shown that infiltration of MΦ into inflamed organs is decreased in the absence of CCR2 (62) (19), and $Gr1^{HI}$ MΦ have been proposed to be CCR2-dependent. To analyze the contribution of CCR2 in the labeling efficiency of circulating monocytes, Lx expression on blood $CD115^{+}$ $F4/80^{+}$ monocytes were analyzed (Fig. 20).

As already observed in CCR2-competent mice (Fig. 18), labeling of circulating $Gr1^{HI}$ monocytes in infected mice was more efficient than labeling of the $Gr1^{LO}$ counterparts. However, labeling of $Gr1^{HI}$ monocytes in CCR2-deficient mice under non-inflammatory conditions was smaller than that of $Gr1^{LO}$ monocytes (Fig. 20), suggesting that CCR2 affected the labeling efficacy of $Gr1^{HI}$ monocytes in the blood. Nevertheless, both MΦ subtypes could specifically be labeled, allowing analysis of their migration pattern in UTI.

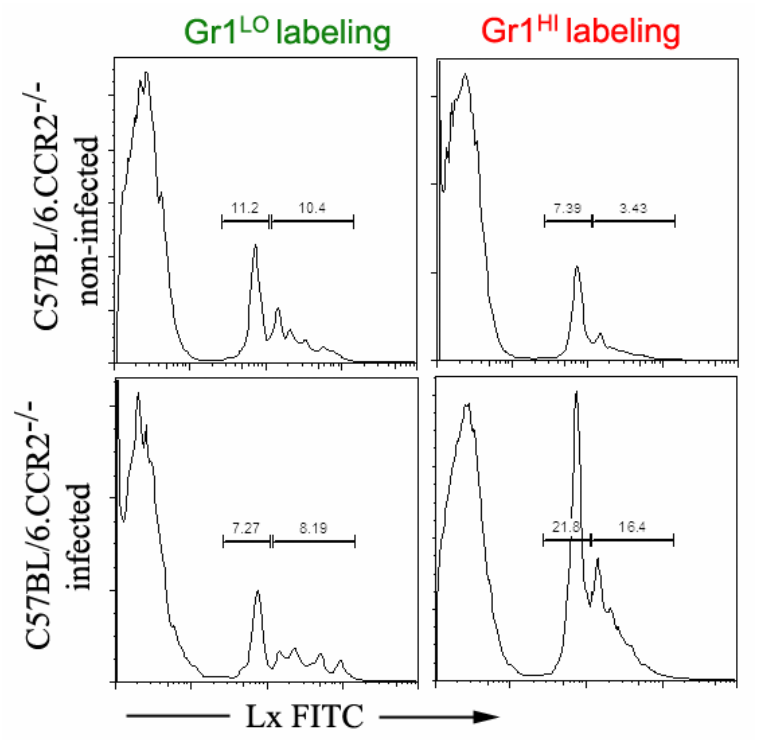


Fig. 20. Influence of CCR2 expression on labeling efficiency of monocyte subsets

Lx beads were administered into CCR2-deficient mice to label either $Gr1^{LO}$ or $Gr1^{HI}$ monocytes. Whole blood was subjected to red cell lysis using RCB buffer and washed twice in DMEM and the expression level of Lx on $CD115^{+}$ $F4/80^{+}$ cells were analyzed. Shown are the mean ± SD of groups of 3 mice. Results are representative of three individual experiments.

7.5.3. $Gr1^{LO}$ and $Gr1^{HI}$ monocytes infiltrate the CCR2-deficient bladder

Labeling of either $Gr1^{LO}$ or $Gr1^{HI}$ monocytes in the circulation of CCR2-deficient mice enabled to monitor the contribution of CCR2 in monocyte trafficking into the bladder. Surprisingly, infiltration of CCR2-deficient $Gr1^{LO}$ and $Gr1^{HI}$ monocytes still occurred in UTI, indicating CCR2 independent migration into infected bladders (Fig. 21A). Nevertheless, the abundance of both MΦ subsets in the infected bladder was significantly decreased in the absence of CCR2 (Fig. 21C). In contrast, CCR2-mediated differences were not observed under homeostasis (Fig. 21B). These findings demonstrated that CCR2 contributed to the abundance of both $Gr1^{LO}$ and $Gr1^{HI}$ precursors in UTI, but was not essential for this process.

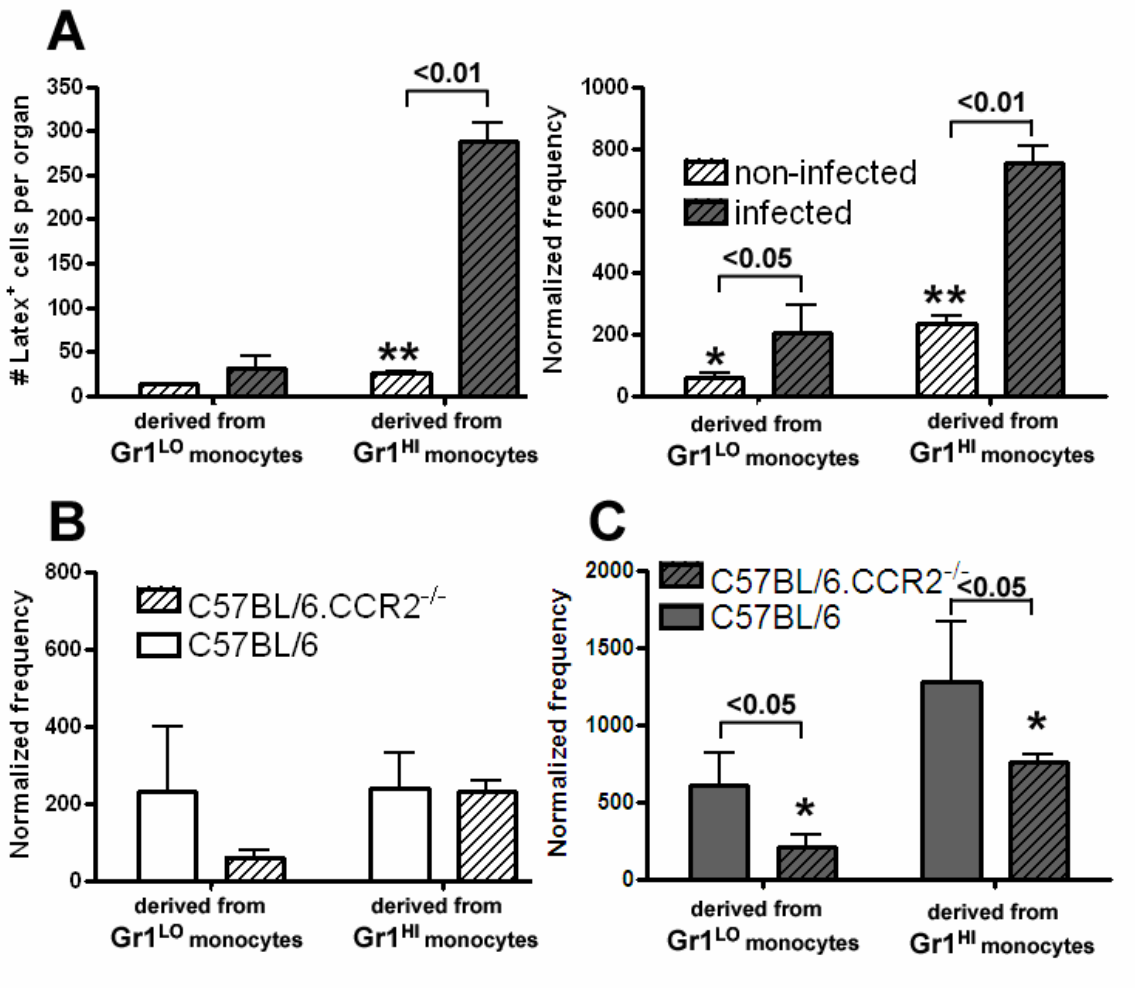


Fig. 21. Monocyte subsets in the bladder

Circulating monocytes were labeled with fluorescent Lx beads. Two days later C57BL/6 and CCR2-deficient mice were infected with $5x10^8$ *E.coli* 536. After 24 hours, the bladders of infected and non-infected control mice were digested with collagenase and stained for CD115, F4/80 and Gr1. Lx expression on CD115 and F4/80 positive cells were analyzed. Shown are the mean ± SD of groups of 5 mice. Results are representative of three individual experiments. One asterisk indicates a p value <0.05, ** p<0.01, *** p<0.001.

7.6. CCR2-dependent migration of monocytes

These results described above could either be due to a role of CCR2 in recruitment of monocytes to the bladder, or to a role of CCR2 in emigration of monocytes from the BM into the blood, as very recently suggested (76). To distinguish between these two possibilities, the composition and abundance of monocytes were analyzed in mice, where the two monocyte subsets were neither depleted nor labeled. To this end, Gr1 expression on $CD115^{+}$ $F4/80^{+}$ monocytes in the BM, the blood and the bladder were analyzed in infected mice and in non-infected control animals (Fig. 22).

In the BM, proportions of monocyte subsets were similar in the presence and absence of CCR2 (Fig. 22). In the blood and the bladder, however, CCR2 significantly affected these proportions. The percentage of blood $Gr1^{HI}$ monocytes were lower in non-infected CCR2-deficient mice. In infection, however, this proportion in the blood was unaltered by CCR2. In the bladder, the percentage of $Gr1^{HI}$ MΦ in infected CCR2-competent mice was markedly increased, whereas CCR2-deficient mice showed a higher proportion of $Gr1^{LO}$ MΦ. These data suggested that CCR2 contributed to the proportion of monocytes in the circulation and this may subsequently affected the MΦ proportions in infected CCR2-deficient bladders.

These differences in the infected CCR2-deficient bladder might be due to increased infiltration of $Gr1^{LO}$ MΦ into the deficient bladder or to abolished infiltration of $Gr1^{HI}$ MΦ. Another possibility could be differences in the composition of blood monocytes, which changed the availability of MΦ precursors.

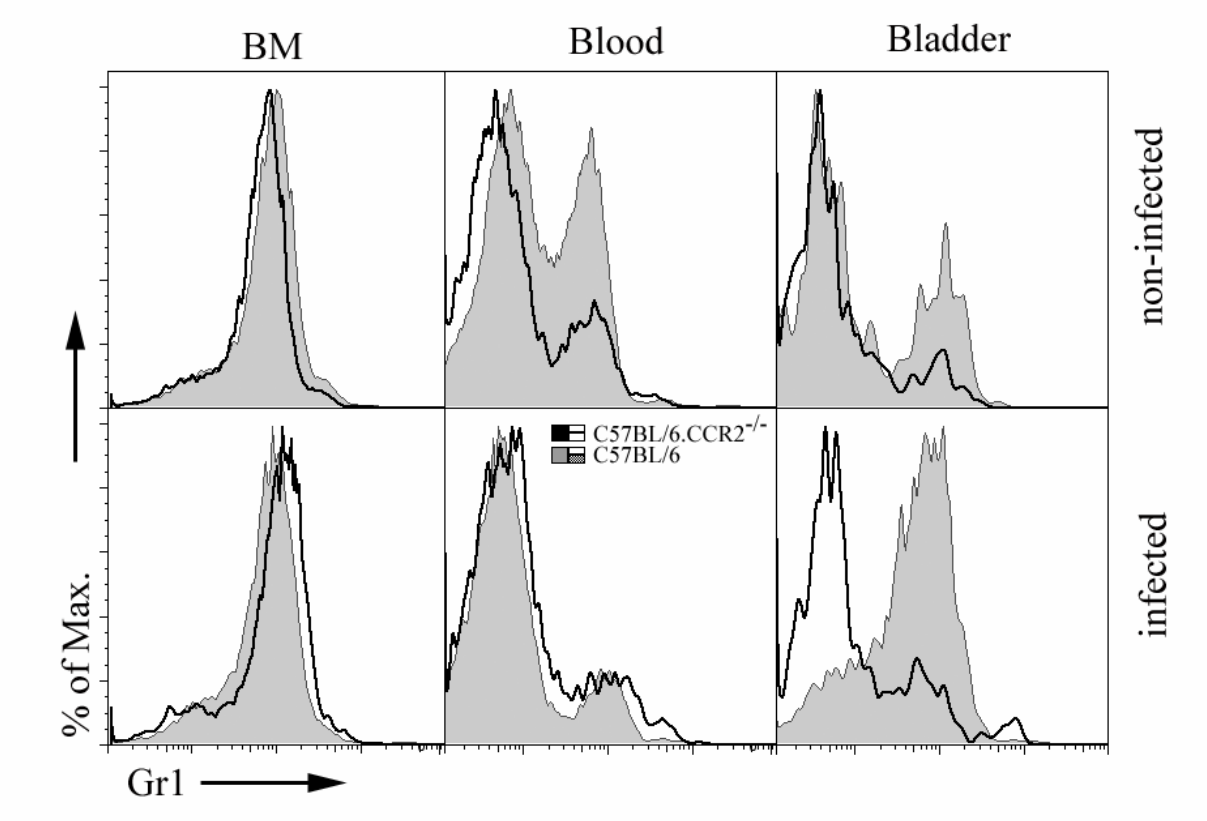


Fig. 22. Gr1 expression on monocytes

C57BL/6 and CCR2-deficient mice were infected with $5x10^8$ *E.coli* 536. After 14 hours, the bladders of infected mice and non-infected controls were digested with collagenase and stained for CD115, F4/80 and Gr1. Gr1 expression on CD115 and F4/80 positive cells were analyzed. Results are representative of 3 individual experiments. Numbers represents the percentage of infected mice.

To discriminate between these possibilities, the proportions of monocyte susbets were determined in these organs and additionally the number of monocytes in bladder were analyzed (Fig. 23). Differences in the proportion of $Gr1^{LO}$ and $Gr1^{HI}$ monocytes in the BM of C57BL/6 and CCR2-deficient mice were not observed. In contrast, the percentage of both monocyte subsets in the circulation and the bladder of infected CCR2-deficient mice were reduced significantly, indicating less abundance of circulating monocytes in CCR2-deficient mice. These data suggested that in addition to its role in immigration of monocytes into infected tissue, CCR2 might contribute to the emigration of monocytes from the BM into the circulation. This led to decreased numbers of monocytes in the blood, which subsequently diminished the number of MΦ in the infected bladder.

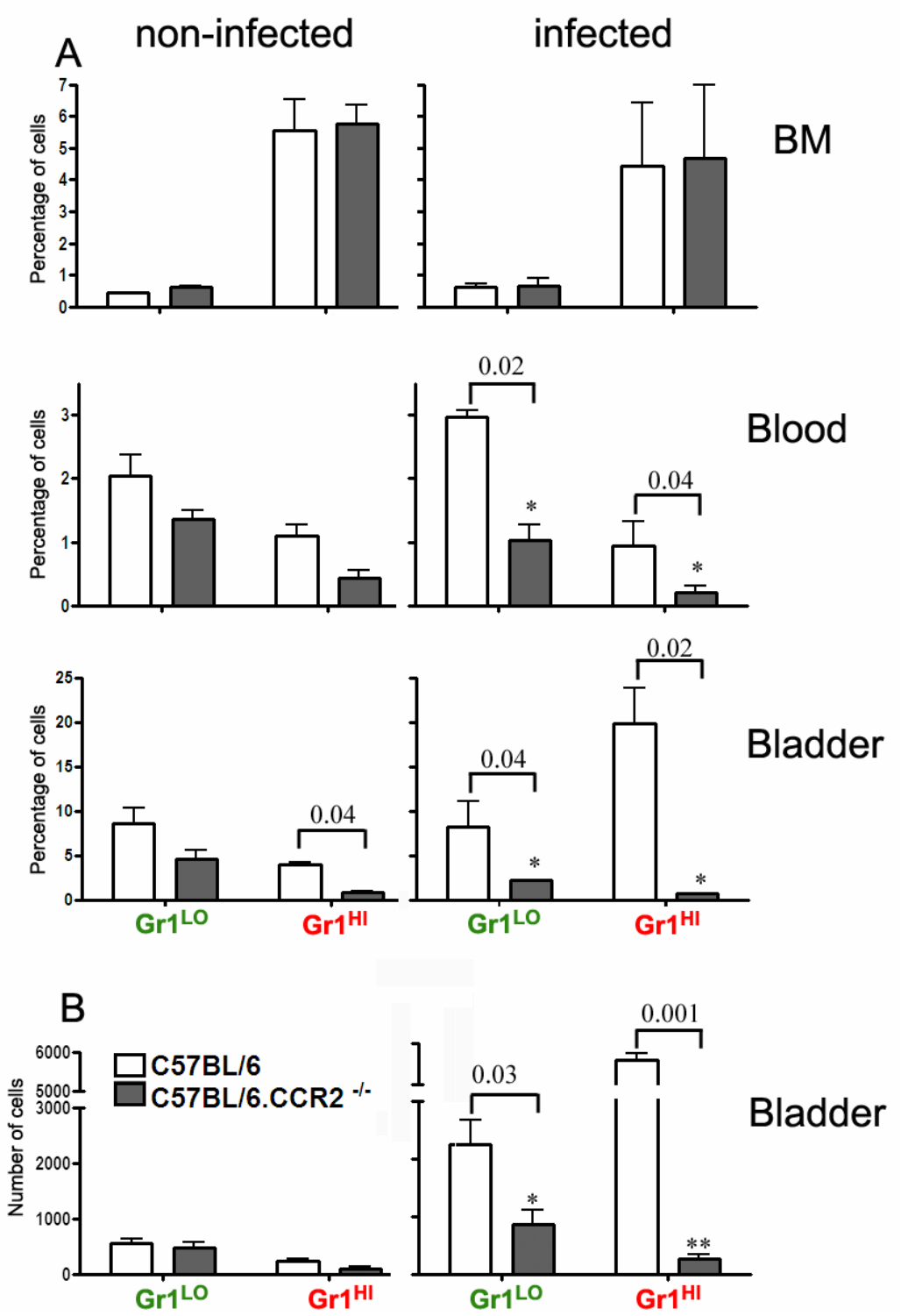


Fig. 23. Proportions and absolute cell numbers in different organs involved in UTI

C57BL/6 and CCR2-deficient mice were infected with 5x10^8 *E.coli* 536. After 14 hours, cells from the BM, the blood and the bladders of infected mice and non-infected controls were isolated and stained for CD115, F4/80 and Gr1. Gr1 expression on CD115 and F4/80 positive cells were analyzed. Shown are the mean ± SD of groups of 5 mice. Brackets with numbers indicate p values where results were statistically significant. Results are representative of 3 individual experiments. One asterisk indicates a p value <0.05, ** p<0.01, *** p<0.001.

8. DISCUSSION

8.1. Recruitment of DC into the bladder after instillation of UPEC

The early defense against bacterial infections is thought to depend mainly on resident tissue cells and innate immune effectors such as NΦG (32) (55). A role of DC as innate effectors has recently been demonstrated in several experimental models (15, 60). In particular, a DC subpopulation termed Tip-DC was critical for the defense against infection with the gram-positive bacterium *Listeria monocytogenes.* Its absence in CCR2-deficient mice resulted in high susceptibility to this infection (77). Based on these findings, the role of DC was studied in bacterial UTI, one of the most prevalent infections world-wide. DC appeared in the bladder as fast as NΦG and MΦ, implying recruitment from the circulation. Expression of F4/80 at intermediate levels and high or low expression levels of CD11b was noted, consistent with previous studies examining the phenotype of DC in other non-lymphoid tissues (41, 62). In addition, a considerable population of DC with phenotypical characteristics of Tip-DC was found, including their absence in CCR2-deficient mice, intermediate expression of CD11b, expression of iNOS and secretion of TNFα. These findings are the first to describe DC recruitment to the bladder in UTI. Furthermore, they represent the first description of Tip-DC in an infection model other than listeriosis.

8.2. CCR2-dependent cells are dispensable for bacterial clearance

The CCR2 ligand, MCP-1 (CCL-2), has been shown to be secreted in cystitis and pyelonephritis (34) (11), suggesting a functional role in these infections. However, bacterial clearance from bladders of infected CCR2-deficient mice was unchanged. This independence of $CCR2^+$ cells indicated that MCP-1 secretion was not essential for innate defense against UTI. Furthermore, it implied that Tip-DC were dispensable for this defense. This discrepancy to the situation in listeriosis may be explained by differences in the immune response elicited by infections with gram-positive *Listeria* and gram-negative *E.coli*. Alternatively, immune mediators that could replace the functionality of Tip-DC may reside in the bladder, but not in the spleen. In support of the latter possibility, vesical TNFα- and iNOS-producing MΦ were abundant in the absence of CCR2, so that the lack of Tip-DC did not significantly deplete the

supply of these immune mediators. TNFα and iNOS have been shown to be crucial in experimental models of other bacterial infections (48) (77). In UTI, both mediators were produced in high amounts (48) (64). However, a functional role in this infection has not been proven, although some studies reported partial dependence on iNOS in mice deficient for this enzyme or upon chemical inhibition of NO (59) (63). The role of TNFα has not been experimentally addressed yet. This cytokine performed pleiotropic functions relevant in infections, such as MΦ activation or recruitment of NΦG (48).The number of NΦG were not reduced in the bladders of CCR2-deficient mice, implying that the factors crucial in their recruitment, such as CXCL8 and TNFα must have been functionally available without Tip-DC. Indeed, vesical TNFα–production was detectable in these animals, albeit at lower levels. However, the demonstration of unaltered clearance of UPEC in Tip-DC-deficient CCR2-deficient bladders indicated dispensability of factors produced by this DC subtype in UTI.

8.3. DC are dispensable for innate clearance, but take up inoculated UPEC

These findings fundamentally questioned the necessity of DC for the innate defense against UTI. Indeed, in mice depleted of DC, UPEC were cleared as efficiently as in non-depleted controls. The dispensability of all DC for bacterial clearance did not exclude a supportive role of these cells in the innate defense against UTI, for example by secretion of proinflammatory cytokines.

Nevertheless, EM revealed cells with dendritic protrusions in the uroepithelium and bladder submucosa, which contained neither the segmented nuclei of NΦG, nor large lysosomes typical of MΦ. The surprisingly early appearance of DC two hours after infection may allow these cells to capture UPEC at an early stage of infection. Using UPECgfp, this uptake was shown by flow-cytometry and microscopically. High levels of MHC class II and costimulatory molecules, in particular on those DC that had phagocytosed UPEC implied a suggestive role in antigen presentation of UPEC. Importantly, expression of these molecules required the presence of CCR2. In the absence of this receptor, MHC class II^+ DC and DC that had phagocytosed UPEC were markedly reduced. It has been suggested that CCR2 may mediate maturation of DC rather than their recruitment (14). In this case, lack of CCR2 would result in an increased population of DC that had been unable to express MHC class II. Indeed, such an increase was found, but it was not statistically significant.

Nevertheless, the efficient recruitment of large numbers of DC bearing an activated and mature phenotype into the bladder uroepithelium was remarkable, and suggests a role in the induction of adaptive immunity. This notion is further supported by the observed uptake of bacterial antigens by DC, which is required for subsequent antigen presentation to T cells. T and B cell-dependent immunity has recently been shown to be able to confer protection in experimental re-infection with UPEC (86).

8.4. Recruitment of $Gr1^{LO}$ and $Gr1^{HI}$ monocytes in the bladder

Infiltration of monocytes into infected and non-infected organs is a well known feature of the innate immune defense. Recent work has shown that monocytes can be subdivided into cells expressing high or low levels of the Gr1 molecule (24). These monocytes have distinct migrational behavior due to distinct expression of chemokine receptors. Circulating $Gr1^{LO}$ monocytes have been associated with precursors for resident tissue MΦ expressing high levels of CX_3CR1, whereas CCR2 expressing $Gr1^{HI}$ monocytes are been described to give rise to inflammatory MΦ, which infiltrate inflamed tissue. The present study showed that $Gr1^{LO}$ monocytes were the predominant MΦ subset in non-infected bladders, whereas $Gr1^{HI}$ MΦ were most prevalent in infected bladders. An increased proportion of the $Gr1^{LO}$ MΦ subset in infection was not observed. However, when the absolute cell numbers in the non-infected and the infected bladder were calculated, a significant increase also of these cells in infection was apparent. These finding illustrates the importance of determining absolute cell numbers, which more accurately reflect the situation in an immune response. Furthermore, these findings question the current view that the $Gr1^{LO}$ MΦ subset is only a resident cell type. Instead, these cells have to be considered also as inflammatory monocytes.

Further analysis of the $Gr1^{LO}$ subset revealed CCR2-independent expression of iNOS exclusively expressed on this MΦ subset. In support of this, the expression of iNOS was not altered in CCR2-deficient mice, although the numbers of $Gr1^{LO}$ MΦ were diminished in these animals. This could be explained by CCR2 independent recruitment of iNOS-expressing $Gr1^{LO}$ MΦ, which represented a minor subpopulation of $Gr1^{LO}$ MΦ. Obviously, only the more abundant iNOS-deficient $Gr1^{LO}$ MΦ infiltrated infected bladders in a CCR2-dependent manner, and this diminished the total number of $Gr1^{LO}$ MΦ in the infected bladder of CCR2-deficient mice significantly. An alternative explanation for the diminished number of

$Gr1^{LO}$ MΦ in CCR2-deficient mice is the conversion of $Gr1^{HI}$ into $Gr1^{LO}$ monocytes. It has been shown by adoptive transfer experiments that circulating $Gr1^{HI}$ monocytes can convert into $Gr1^{LO}$ monocytes. This conversion might also take place in the bladder and the lack of $Gr1^{HI}$ MΦ in the CCR2-deficient bladder might contribute to the diminished numbers of $Gr1^{LO}$ MΦ in the non-infected and infected bladder. Because $Gr1^{LO}$ MΦ infiltrated the bladder in UTI, and because they were involved in executing immune effector functions, I suggest replacing the term "resident" MΦ by the more accurate phrase "patroling" MΦ.

8.5. CCR2 is involved in egress of monocytes from the BM into the blood

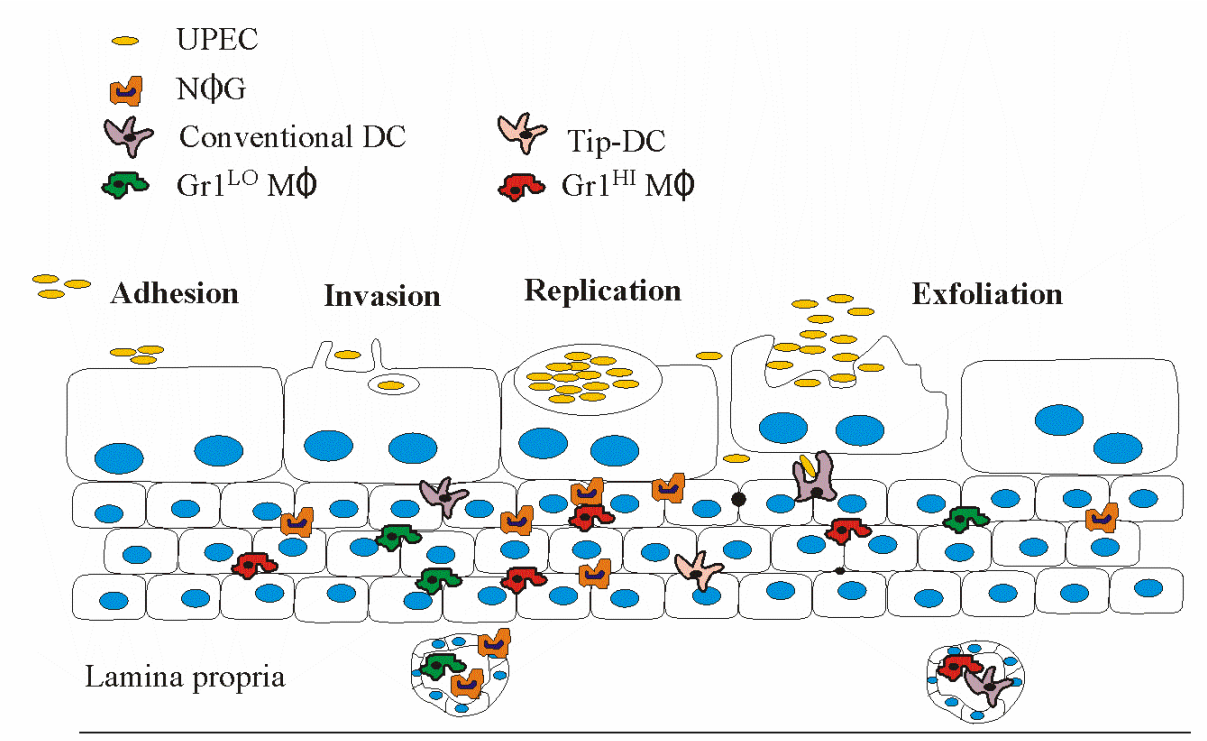
Migration of leukocytes from the BM into the circulation is a well regulated process. Under non-inflammatory conditions, the chemokine CXCR4 has been shown to mediate homeostatic emigration of monocytes from the BM (9, 56) (61). Furthermore, less retention of leukocytes in the BM by diminished interaction of SDF-1 and CXCR4 and therefore diminished interactions with members of the integrin family regulate the homeostasis in infected organisms. In infections, the numbers of circulating NΦG rise dramatically and this has been shown to be mediated by CXCL8, formerly known as IL-8 and MIP-2. Also monocyte numbers rise due to regulated egress of precursors from the BM, and this has been very recently proposed to be mediated by MCP-1 (CCL-2) (76). Migration of circulating monocytes into infected organs is though to be driven by chemotactic signals and their receptors on several target cells. CCR1, CCR2 and CCR5 have been shown to contribute to this process. Since MCP-1 is produced in the urogenital tract in response to bacterial infection, the role of the MCP-1 receptor, CCR2, in migration of monocytes was investigated. To this end, a recently described technique to selectively label $Gr1^{LO}$ and $Gr1^{HI}$ monocytes in the blood was employed (84). Different labelling efficiency of the $Gr1^{LO}$ and the $Gr1^{HI}$ subset had to be taken into account. These differences may be explained by lower uptake of the Lx beads by the $Gr1^{LO}$ monocyte subset or by a decreased ratio of cells to Lx beads after depletion of the $Gr1^{LO}$ monocyte subset. In conclusion, the labelling technique revealed CCR2-independent migration of both $Gr1^{LO}$ and $Gr1^{HI}$ monocytes into infected tissue, suggesting that additional mechanisms also contributed to their recruitment, for example CCR1 and CCR5, which have been described to mediate monocyte infiltration into inflamed organs (33, 46).

Under homeostasis, CCR2-deficient mice showed decreased proportion of $Gr1^{HI}$ monocytes in the blood and the bladder. In contrast, under infectious conditions the proportion was decreased in the bladder of CCR2-deficient mice, but unchanged in the blood. These differences in the blood under homeostasis and infectious conditions were due to recruitment of blood monocytes from the circulation into the bladder after infection. This view has been extended by very recent investigations by others showing that CCR2 mediates egress of monocytes out of the BM, rather than from blood to site infection (76). The contribution of CCR2 in egress of monocytes out of the BM has not been proven directly in this present study, but the altered number and proportion of blood monocytes in CCR2-deficient mice suggested that CCR2 contributed to the egress of monocytes out of the BM. Thus, the present findings in non-infected and infected CCR2-deficient mice are more compatible with this very recent view by others (76).

Taken together, these findings suggested that decreased or altered monocyte proportions and abundance in the infected CCR2-deficient bladder is rather due to decreased abundance of blood monocytes in the circulation of CCR2-deficient mice than from diminished recruitment of monocytes from the bloodstream into the bladder.

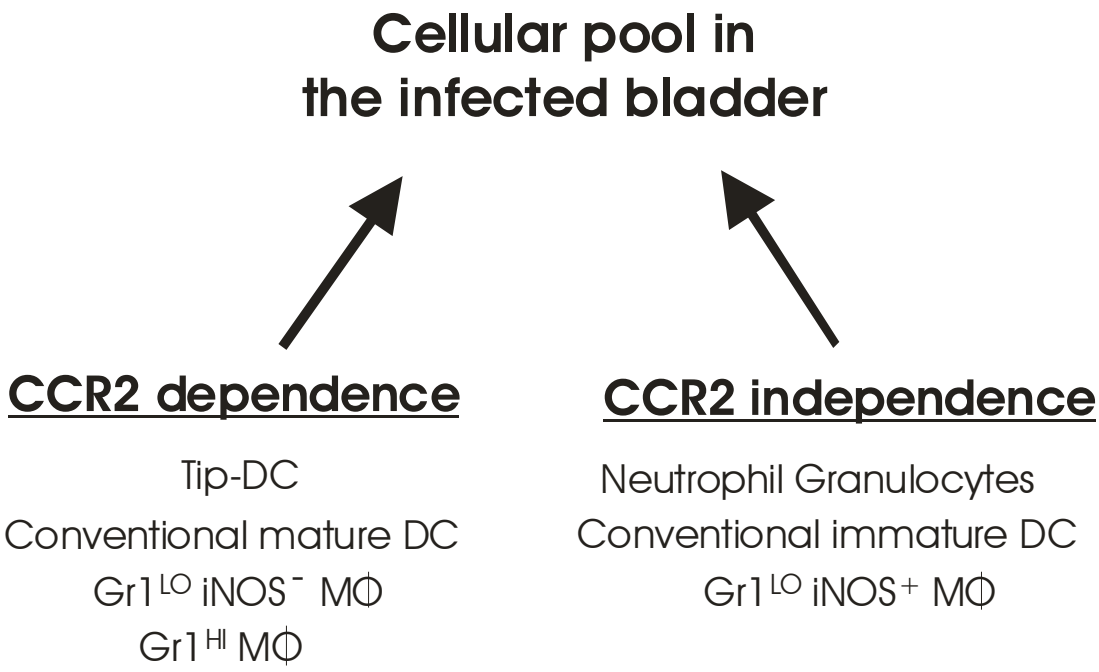
8.6. New model of phagocyte infiltration and migration

The results of the present study warrant extending the current view of cellular infiltration in UTI (scheme 1) by a new model shown in Scheme 4. The abundance of vesical phagocytes could be shown to be comprised of five, rather than the traditional two subsets (MΦ and NΦG): Infiltrating MΦ were comprised of "patroling" $Gr1^{LO}$ and "inflammatory" $Gr1^{HI}$ MΦ. Furthermore, two subsets of DC were identified, which could be subdivided in Tip-DC and cDC.



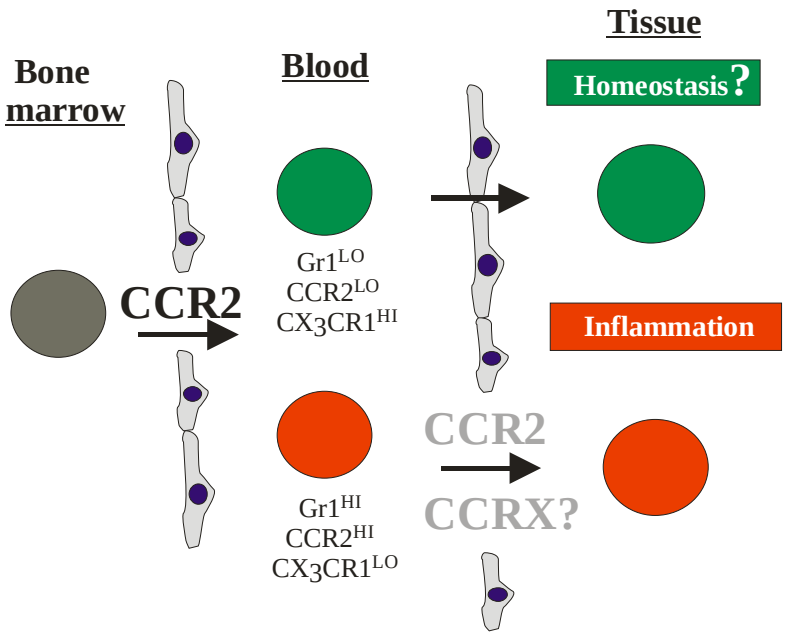
Scheme 4. Interplay between infiltrating cells and UPEC infection

Typical functions of CCR2-dependent Tip-DC were the secretion of innate immune effectors like iNOS and TNFα, whereas cDC were capable of phagocytosis and expressed high levels of MHC class II and costimulatory molecules in a CCR2-dependent manner on their surface (Scheme 5). $Gr1^{LO}$ MΦ were comprised of CCR2-dependent iNOS-negative and CCR2-independent iNOS-producing cells. A typical function of CCR2-dependent $Gr1^{HI}$ MΦ was the expression of TNFα, whereas CCR2-independent NΦG was the most phagocytic cell type. Interestingly, all hematopoietic cells examined produced β1-defensins, a function previously ascribed only to epithelial cells (35).



Scheme 5. CCR2-dependent and independent cell subsets in the infected bladder

The role of CCR2 in phagocyte migration described in the introduction (scheme 2) may be extended by the following novel scheme (Scheme 6): These present findings support mediating egress from the BM as the major function of CCR2. Furthermore, they demonstrate a minor role of CCR2 also in immigration of both $Gr1^{LO}$ and $Gr1^{HI}$ monocytes into infected organs and a role of other CCR in this migration process.



Scheme 6. The role of CCR2 in leukocyte migration

8.7. Future questions to be addressed

This present study warrants future experiments about MΦ and DC under homeostasis and infectious conditions. Questions to be addressed in the future could concern the origin of vesical MΦ and DC in UTI, because it is unknown whether these cells were derived from the same blood precursor, as recently proposed (21). Furthermore, the possibilities of interconversion of DC and MΦ and of $Gr1^{HI}$ MΦ to $Gr1^{LO}$ MΦ need to be addressed. The different functional relevance of the two MΦ subsets in the defense against UTI has to be established. Likewise, the exact role of CCR2 and its ligand in release of monocytes from the BM needs further investigation. The finding that MΦ could be recruited by mechanisms other than CCR2 warrants identification of these mediators. Possible candidates include CCR1 and CCR5.

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11. APPENDIX

PUBLICATIONS

1. T. Kruger, D. Benke, F. Eitner, A. Lang, M. Wirtz, E. E. Hamilton-Williams, **D. Engel**, B. Giese, G. Muller-Newen, J. Floege and C. Kurts (2004). "Identification and Functional Characterization of Dendritic Cells in the Healthy Murine Kidney and in Experimental Glomerulonephritis." J Am Soc Nephrol 15(3): 613-21.

2. A. Lang, D. Benke, F. Eitner, **D. Engel**, S. Ehrlich, M. Breloer, E. Hamilton-Williams, S. Specht, A. Hoerauf, J. Floege, A. von Bonin and C. Kurts (2005). "Heat Shock Protein 60 Is Released in Immune-Mediated Glomerulonephritis and Aggravates Disease: In Vivo Evidence for an Immunologic Danger Signal." J Am Soc Nephrol 16(2): 383-91.

3. **D. Engel**, U. Dobrindt, A. Tittel, P. Peters, J. Maurer, I. Gütgemann, B. Kaissling, W. Kuziel, S. Jung, C. Kurts (2006). "TNFα/iNOS-producing dendritic cells are rapidly recruited to the bladder in urinary tract infection, but are dispensable for bacterial clearance" Infection and immunity, 74(11):6100-7.

POSTER

1. **D. Engel**, U. Dobrindt, C. Kurts (Maastricht, 2004). "Role of Dendritic Cells in Ascending Urinary Tract Infection"

2. **D. Engel**, U. Dobrindt, P. Peters, S. Jung, W. Kuziel, C. Kurts (Kiel, 2005). Dendritic Cells in the early defense against Urinary Tract Infection

3. **D. Engel**, U. Dobrindt, P. Peters, S. Jung, W. Kuziel, C. Kurts (Paris, 2006). TNFα/iNOS-producing dendritic cells are rapidly recruited into the bladder, but are dispensable for bacterial clearance