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Damage-associated Molecular Patterns, Commensals and Pathogens in Renal Pathology

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Faculteit der Geneeskunde

to the memory of my father,

to my mother

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Chapter 1

General Introduction and outline of this thesis

General introduction

Basics of renal anatomy and physiology

The two bean-shaped organ, located on either side of the spine in the retroperitoneal space are approximately 11 cm long, 6 cm wide and 3-5 cm thick, weighing 115-175 grams. The primary function of this complex organ is to maintain body homeostasis via a highly specialized and structured microscopic anatomy. The smallest functional unit of a kidney is called a nephron. A healthy adult human kidney consists of approximately 1 million nephrons. Each nephron comprises a glomerulus and a tubular compartment (proximal tubule, Henle's loop, distal tubule and collecting duct). Circulating blood is filtered by the glomerulus which is surrounded by Bowman's capsule that makes the connection to the tubular compartment[1]. The resulting filtrate, free of cells and large proteins, flows through the different parts of the tubule where reabsorption of water, salts and other essential molecules takes place, while waste products and toxins are secreted in the collecting duct. After entering collecting duct, urine passes through the ureter into the bladder. Filtration, reabsorption and secretion by the kidneys, keep the body in balance in regard to water, minerals, electrolytes and hydrogen ion concentration. Apart from this, kidneys serve as an endocrine organ, by producing erythropoietin, renin, prostaglandins and vitamin D3.

Renal diseases relevant for this thesis

Urinary tract infection

The therapy of choice for patients with end stage renal disease (ESRD) is kidney transplantation. Successful renal transplantation depends on a life-long use of adequate immunosuppression in order to prevent graft rejection. Immunocompromised patients such as renal transplant patients are vulnerable to infectious complications post transplantation[2-5]. Urinary tract infections (UTIs) are the most common bacterial infections with a prevalence of 60% during the first year post renal transplantations[6]. The predominant isolated pathogens are *Escherichia coli (E. coli)*, *Enterococcus* and *Staphylococcus*[2, 4, 5]. Female gender, advanced age, cadaveric donor, acute rejection episodes and greater degrees of immunosuppression are risk factors for UTI in renal transplant recipients[3, 7, 8]. UTIs can often lead to acute graft pyelonephritis, which is considered a potential risk factor for poorer graft function[9, 10]. First month post-transplantation bacteremia associated mortality is 11%[11]. UTIs are the most

common source of sepsis in renal transplant recipients[12]. The standard use of trimethoprim-sulfamethoxazole prophylaxis within the first 6 month after renal transplantation seems to be ineffective to prevent the high incidence and harm of UTIs[13]. Use of routine prophylaxis TMP-SMZ in renal transplant patients has been associated with an increasing amount of resistant *E. coli* isolates against TMP-SMX[13].

UTI can be studied in an experimental mouse model whereby infection is initiated by transurethral inoculation of *E. coli* suspension into the bladder. Bacterial outgrowth and inflammatory response can be determined 24 and 48 hours later. This model has been used in chapter 2 of this thesis, where we investigated the potential effects of Tacrolimus on host immune response.

Acute Kidney Injury

Acute kidney injury (AKI), a sudden decline in kidney function reflected by elevated serum levels of creatinine and urea[14], is a common clinical complication which affects 5% of hospitalized patients. AKI associated mortality rate is 50-80% in critically ill patients[15].

Ischemia reperfusion (I/R)-injury is the predominant cause of AKI, occurring during shock and transplantation[16]. I/R-injury is defined as an abrupt transient drop in blood flow, depriving kidneys from nutrients and oxygen followed by restoration of blood flow, the reperfusion phase[17]. Ischemia induces cell death of renal tubular epithelial cells (TECs) either via mechanism of apoptosis or necrosis[18], resulting in local release of damage-associated molecular patterns (DAMPs) such as HMGB1, heat shock proteins and mitochondrial DNA[19-22]. TECs however are not only passively damaged by injury but also actively participate in the inflammatory response[18]. DAMPs activate TECs and immune cells via PRRs, initiating a state of inflammation which is the hallmark in AKI. The early immune response during I/R-injury is initiated by TECs and renal resident macrophages releasing pro-inflammatory mediators such as TNF-α, MCP-1 and IL-8[19, 20, 22, 23]. Subsequently, one day after I/R-injury the inflammatory milieu is predominantly characterized by a vast influx of granulocytes[24], while in a later phase macrophages and lymphocytes become more prominent[25]. This state of sterile inflammation upon I/R-injury is recognized as a mechanism with a double-edge sword. Certainly, initiation of an inflammatory response is of crucial importance in recovering tissue damage. However, prolonged and excessive inflammation may cause more harm eventually leading to renal fibrosis and end-stage renal disease (ESDR)[26].

I/R-injury can be studied in an experimental animal model whereby unilateral or bilateral renal arteries are clamped with micro aneurysm clamps for a specific period of time. This model has been used in chapter 3 and 4 of this thesis, where we studied the impact of gut microbiota and platelets during I/R-injury respectively.

Chronic kidney disease

Chronic kidney disease (CKD) with a global prevalence of 12% is a major public health burden associated with reduced quality of life, high morbidity and mortality[27]. CKD is a general term for kidney diseases with variable etiology affecting renal structure and thereby its function. Criteria defining CKD include albuminuria and a glomerular filtration rate (GFR) of < 60 ml/min/1.73 m^2 for a period longer than 3 month. Loss of nephrons due to advanced age, acute or chronic kidney injuries caused by toxic exposures or diseases including UTI, AKI, diabetes mellitus type 2, obesity and hypertension are risk factors for development and progression of CKD.

Renal fibrosis is the final common pathway for all types of CKD, leading to ESRD, an irreversible condition that requires renal replacement therapy[28]. Unilateral ureteral obstruction (UUO) in mice is an often-used model to investigate mechanisms of renal fibrosis. UUO model mimics chronic obstructive nephropathy which is a major cause of renal insufficiency in children. UUO model induces several characteristics of renal fibrosis, including tubulointerstitial inflammation, myofibroblast accumulation, increased deposition of extracellular matrix (ECM) and tubular atrophy. The majority of infiltrating leukocytes are macrophages which play a dual role by either promoting fibrosis or resolving tissue injury in a later stage[29]. Macrophages promote fibrosis mainly by release of transforming growth factor-β (TGFβ), which generates ECM deposition by increasing protein synthesis and decreasing matrix protein degradation[30]. In a later stage of the injury, macrophages are involved in phagocytosis of excessive ECM fragments and promote hereby tissue repair[31]. Macrophages are also involved in promoting TEC apoptosis which together with declined TEC proliferation shifts the balance towards cell death, eventually resulting in tubulointerstitial atrophy[32, 33]. In Chapter 5 of this thesis we used the UUO model to unravel the role of NOD1/2 in renal injury and fibrosis.

Pattern recognition receptors

As discussed previously in this introduction, inflammatory responses are the underlying cause of many renal diseases[19]. The innate immune response against invading pathogens and endogenous DAMPs has a crucial role in induction, amplification and resolution of (renal) injury. This process requires involvement of highly conserved germline-encoded PRRs[19]. Importantly, inflammatory responses can be divided into sterile inflammation (e.g. during AKI and CKD) and pathogen-induced inflammation (e.g. UTI).

Toll-like receptors

TLR family is the most extensively studied PRRs, consisting of 13 different members in mice and 10 in humans[34]. All TLRs have an extracellular recognition domain consisting of leucine-rich repeats and a cytoplasmic Toll/IL-1 Receptor (TIR) domain. TLRs can be activated by pathogen-associated molecular patterns (PAMPs) or DAMPs, subsequently initiating a downstream signaling pathway[35, 36]. Via MyD88-dependent pathway TLR activation results in phosphorylation of Iκβ, resulting in nuclear translocation of NF-κβ. Activation of NF-κβ leads to release of pro-inflammatory mediators generating an inflammatory milieu[37]. Several TLRs have been shown to play a crucial role in pathogenesis of many renal diseases[19]. For instance, TLR11, TLR4 and TLR5 have been described to play a role in the immune defense against UTIs *in vivo*[38-44].

NOD-like receptors

NOD1 and NOD2 belong to the intracellular family of nucleotide-binding oligomerization domain receptors (NLRs). NODs are expressed on a wide variety of cell types, including cells of the innate immunity but also on epithelial cells of different tissues (e.g. lung, intestinal, kidney)[19]. Specific substructure from bacterial cell wall, peptidoglycan (PGN) is sensed by NOD receptors; gram negative derived PGN containing (diaminopimelic acid) DAP by NOD1 and muramyl dipeptide (MDP) from gram positive and negative bacteria by NOD2[45-48]. The downstream signaling pathway includes engagement of CARD-containing kinase RIP2, a protein that interacts with NOD1 and NOD2 via CARD-CARD interactions resulting in activation of NF-κβ and MAPKs[49, 50]. To date, no endogenous DAMPs have been identified that signal through NODs. However, participation of NOD1 and NOD2 has been discovered in sepsis- ischemia-induced AKI, suggesting a potential DAMP (released upon ischemic

damage) that signals through these receptors[51, 52]. In Chapter 5, we studied the role of NOD1/2 in renal injury and fibrosis induced by ureteral obstruction.

Immunosuppression in renal transplantation

The therapy of choice for ESRD is renal transplantation, which is superior compared to dialysis in terms of survival, quality of life and cost effectiveness. The ultimate goal of immunosuppression post transplantation is to prevent graft rejection with minimum undesired side effects such as infections and drug toxicity. In order to achieve this goal, combination of drugs with different mechanism of action are used simultaneously at relatively low doses. Prevention of graft rejection is based on suppression of lymphocytes, either by diverting lymphocyte traffic or blocking lymphocyte response pathways. The conventional therapy consists of triple-drug therapy including a calcineurin inhibitor (Cyclosporine/Tacrolimus) or a mTOR inhibitor (Sirolimus/Everolimus), an antiproliferative agent (Mycophenolate mofetil), and a corticosteroid (Prednisone). Immunosuppressive regime in transplant patients consists of an induction and a maintenance phase. During the induction therapy, immunosuppression level is at its highest, gradually decreasing during the maintenance phase as the risk of acute rejection declines[53, 54].

Calcineurin inhibitors

Since the discovery of Cyclosporine in the lab of Sandoz in Switzerland in 1972, patients and graft survival following solid-transplantation have improved significantly[55]. However, its side effect causing acute and chronic nephrotoxicity led to discovery of the alternative calcineurin inhibitor Tacrolimus. Tacrolimus was introduced in 1994 and is since widely used in solid organ transplant patients[56]. Tacrolimus, formerly known as FK506, is a macrolide antibiotic from *Streptomyces tsukubaensis* with immunosuppressive properties[57]. Calcineurin is a phosphatase involved in dephosphorylation of nuclear factor of activated T cells (NFAT), which is a transcription factor required for cytokine production by T cells. Calcineurin inhibition impairs translocation of NFAT into the nucleus, resulting in suppression of IL-2, the cytokine that is responsible for lymphocyte activation[58]. Next to its effects on adaptive immunity, calcineurin inhibitors also interfere with innate immunity, an undesired side effect.

Calcineurin is a negative regulator of the TLR-mediated activation pathway by interacting with MyD88, TRIF, TLR2 and TLR4[59]. Therefore, inhibition of calcineurin by Tacrolimus would result in endotoxin tolerance, a state of immune tolerance in cells of the innate immunity such as macrophages. Given the high susceptibility to UTIs in renal transplant patients and the interaction of Tacrolimus with TLR-signaling[60-65], in Chapter 2 we investigated the potential effects of Tacrolimus on host immune response against UTIs.

Outline of this thesis

Given the high susceptibility to UTIs post renal transplantation, in **chapter 2**, we aimed to investigate the potential effect of calcineurin inhibitor Tacrolimus on host immune response during UTI. In **chapter 3**, we reveal mechanistic insights into the gut-kidney axis during AKI. The impact of microbiome in shaping the immune system outside the gut has been intensively studies in the past decades. Several studies discovered that gut microbiome affect the immune response to different inflammatory disorders, however its role in AKI was unknown. In **chapter 4**, we investigated the mechanism behind platelet activation during I/R-injury and how platelet activation contributes to tissue injury. Several PRRs have been described to have a central role in CKD, role of NOD1/2 receptors remained unknown. Therefore, in **chapter 5** we investigated the role of NOD1 and NOD2 in chronic renal inflammation, injury and fibrosis by subjecting WT and NOD1/2 DKO mice to UUO model.

References

1. Nyengaard, JR, Bendtsen, TF: Glomerular number and size in relation to age, kidney weight, and body surface in normal man. *The Anatomical record,* 232**:** 194-201, 1992.
2. Chuang, P, Parikh, CR, Langone, A: Urinary tract infections after renal transplantation: a retrospective review at two US transplant centers. *Clinical transplantation,* 19**:** 230-235, 2005.
3. Fishman, JA, Rubin, RH: Infection in organ-transplant recipients. *The New England journal of medicine,* 338**:** 1741-1751, 1998.
4. Golebiewska, J, Debska-Slizien, A, Komarnicka, J, Samet, A, Rutkowski, B: Urinary tract infections in renal transplant recipients. *Transplantation proceedings,* 43**:** 2985-2990, 2011.
5. Rubin, RH: Infectious disease complications of renal transplantation. *Kidney international,* 44**:** 221-236, 1993.
6. Veroux, M, Giuffrida, G, Corona, D, Gagliano, M, Scriffignano, V, Vizcarra, D, Tallarita, T, Zerbo, D, Virgilio, C, Sciacca, A, Cappello, D, Stefani, S, Veroux, P: Infective complications in renal allograft recipients: epidemiology and outcome. *Transplantation proceedings,* 40**:** 1873-1876, 2008.
7. Camargo, LF, Esteves, AB, Ulisses, LR, Rivelli, GG, Mazzali, M: Urinary tract infection in renal transplant recipients: incidence, risk factors, and impact on graft function. *Transplantation proceedings,* 46**:** 1757-1759, 2014.
8. de Souza, RM, Olsburgh, J: Urinary tract infection in the renal transplant patient. *Nature clinical practice Nephrology,* 4**:** 252-264, 2008.
9. Rice, JC, Peng, T, Kuo, YF, Pendyala, S, Simmons, L, Boughton, J, Ishihara, K, Nowicki, S, Nowicki, BJ: Renal allograft injury is associated with urinary tract infection caused by Escherichia coli bearing adherence factors. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons,* 6**:** 2375-2383, 2006.
10. Abbott, KC, Swanson, SJ, Richter, ER, Bohen, EM, Agodoa, LY, Peters, TG, Barbour, G, Lipnick, R, Cruess, DF: Late urinary tract infection after renal transplantation in the United States. *American journal of kidney diseases : the official journal of the National Kidney Foundation,* 44**:** 353-362, 2004.
11. Linares, L, Garcia-Goez, JF, Cervera, C, Almela, M, Sanclemente, G, Cofan, F, Ricart, MJ, Navasa, M, Moreno, A: Early bacteremia after solid organ transplantation. *Transplantation proceedings,* 41**:** 2262-2264, 2009.
12. Tolkoff-Rubin, NE, Rubin, RH: Urinary tract infection in the immunocompromised host. Lessons from kidney transplantation and the AIDS epidemic. *Infectious disease clinics of North America,* 11**:** 707-717, 1997.
13. Singh, R, Bemelman, FJ, Hodiamont, CJ, Idu, MM, Ten Berge, IJ, Geerlings, SE: The impact of trimethoprim-sulfamethoxazole as Pneumocystis jiroveci pneumonia prophylaxis on the occurrence of asymptomatic bacteriuria and urinary tract infections among renal allograft recipients: a retrospective before-after study. *BMC infectious diseases,* 16**:** 90, 2016.
14. Mehta, RL, Kellum, JA, Shah, SV, Molitoris, BA, Ronco, C, Warnock, DG, Levin, A: Acute Kidney Injury Network: report of an initiative to improve outcomes in acute kidney injury. *Critical care (London, England),* 11**:** R31, 2007.
15. Gill, N, Nally, JV, Jr., Fatica, RA: Renal failure secondary to acute tubular necrosis: epidemiology, diagnosis, and management. *Chest,* 128**:** 2847-2863, 2005.
16. Le Dorze, M, Legrand, M, Payen, D, Ince, C: The role of the microcirculation in acute kidney injury. *Current opinion in critical care,* 15**:** 503-508, 2009.
17. Devarajan, P: Update on mechanisms of ischemic acute kidney injury. *Journal of the American Society of Nephrology : JASN,* 17**:** 1503-1520, 2006.
18. Padanilam, BJ: Cell death induced by acute renal injury: a perspective on the contributions of apoptosis and necrosis. *American journal of physiology Renal physiology,* 284**:** F608-627, 2003.

19. Leemans, JC, Kors, L, Anders, HJ, Florquin, S: Pattern recognition receptors and the inflammasome in kidney disease. *Nature reviews Nephrology,* 10**:** 398-414, 2014.
20. Leemans, JC, Stokman, G, Claessen, N, Rouschop, KM, Teske, GJ, Kirschning, CJ, Akira, S, van der Poll, T, Weening, JJ, Florquin, S: Renal-associated TLR2 mediates ischemia/reperfusion injury in the kidney. *The Journal of clinical investigation,* 115**:** 2894-2903, 2005.
21. Jansen, MP, Emal, D, Teske, GJ, Dessing, MC, Florquin, S, Roelofs, JJ: Release of extracellular DNA influences renal ischemia reperfusion injury by platelet activation and formation of neutrophil extracellular traps. *Kidney international,* 91**:** 352-364, 2017.
22. Pulskens, WP, Teske, GJ, Butter, LM, Roelofs, JJ, van der Poll, T, Florquin, S, Leemans, JC: Toll-like receptor-4 coordinates the innate immune response of the kidney to renal ischemia/reperfusion injury. *PloS one,* 3**:** e3596, 2008.
23. Bakker, PJ, Butter, LM, Claessen, N, Teske, GJ, Sutterwala, FS, Florquin, S, Leemans, JC: A tissue-specific role for Nlrp3 in tubular epithelial repair after renal ischemia/reperfusion. *The American journal of pathology,* 184**:** 2013-2022, 2014.
24. Bolisetty, S, Agarwal, A: Neutrophils in acute kidney injury: not neutral any more. *Kidney international,* 75**:** 674-676, 2009.
25. Dessing, MC, Tammaro, A, Pulskens, WP, Teske, GJ, Butter, LM, Claessen, N, van Eijk, M, van der Poll, T, Vogl, T, Roth, J, Florquin, S, Leemans, JC: The calcium-binding protein complex S100A8/A9 has a crucial role in controlling macrophage-mediated renal repair following ischemia/reperfusion. *Kidney international,* 87**:** 85-94, 2015.
26. Zager, RA: Progression from acute kidney injury to chronic kidney disease: clinical and experimental insights and queries. *Nephron Clinical practice,* 127**:** 46-50, 2014.
27. Hill, NR, Fatoba, ST, Oke, JL, Hirst, JA, O'Callaghan, CA, Lasserson, DS, Hobbs, FD: Global Prevalence of Chronic Kidney Disease - A Systematic Review and Meta-Analysis. *PloS one,* 11**:** e0158765, 2016.
28. Wynn, TA: Common and unique mechanisms regulate fibrosis in various fibroproliferative diseases. *The Journal of clinical investigation,* 117**:** 524-529, 2007.
29. Sean Eardley, K, Cockwell, P: Macrophages and progressive tubulointerstitial disease. *Kidney international,* 68**:** 437-455, 2005.
30. Diamond, JR, Kees-Folts, D, Ding, G, Frye, JE, Restrepo, NC: Macrophages, monocyte chemoattractant peptide-1, and TGF-beta 1 in experimental hydronephrosis. *The American journal of physiology,* 266**:** F926-933, 1994.
31. Nishida, M, Okumura, Y, Fujimoto, S, Shiraishi, I, Itoi, T, Hamaoka, K: Adoptive transfer of macrophages ameliorates renal fibrosis in mice. *Biochemical and biophysical research communications,* 332**:** 11-16, 2005.
32. Ma, FY, Liu, J, Kitching, AR, Manthey, CL, Nikolic-Paterson, DJ: Targeting renal macrophage accumulation via c-fms kinase reduces tubular apoptosis but fails to modify progressive fibrosis in the obstructed rat kidney. *American journal of physiology Renal physiology,* 296**:** F177-185, 2009.
33. Truong, LD, Sheikh-Hamad, D, Chakraborty, S, Suki, WN: Cell apoptosis and proliferation in obstructive uropathy. *Seminars in nephrology,* 18**:** 641-651, 1998.
34. Kumagai, Y, Takeuchi, O, Akira, S: Pathogen recognition by innate receptors. *Journal of infection and chemotherapy : official journal of the Japan Society of Chemotherapy,* 14**:** 86-92, 2008.
35. Tsan, MF, Gao, B: Endogenous ligands of Toll-like receptors. *Journal of leukocyte biology,* 76**:** 514-519, 2004.
36. Johnson, GB, Brunn, GJ, Platt, JL: Activation of mammalian Toll-like receptors by endogenous agonists. *Critical reviews in immunology,* 23**:** 15-44, 2003.
37. Kawai, T, Akira, S: TLR signaling. *Cell death and differentiation,* 13**:** 816-825, 2006.
38. Andersen-Nissen, E, Hawn, TR, Smith, KD, Nachman, A, Lampano, AE, Uematsu, S, Akira, S, Aderem, A: Cutting edge: Tlr5-/- mice are more susceptible to Escherichia coli urinary tract infection. *Journal of immunology (Baltimore, Md : 1950),* 178**:** 4717-4720, 2007.

39. Feuillet, V, Medjane, S, Mondor, I, Demaria, O, Pagni, PP, Galan, JE, Flavell, RA, Alexopoulou, L: Involvement of Toll-like receptor 5 in the recognition of flagellated bacteria. *Proceedings of the National Academy of Sciences of the United States of America,* 103**:** 12487-12492, 2006.
40. Hawn, TR, Scholes, D, Li, SS, Wang, H, Yang, Y, Roberts, PL, Stapleton, AE, Janer, M, Aderem, A, Stamm, WE, Zhao, LP, Hooton, TM: Toll-like receptor polymorphisms and susceptibility to urinary tract infections in adult women. *PloS one,* 4**:** e5990, 2009.
41. Hayashi, F, Smith, KD, Ozinsky, A, Hawn, TR, Yi, EC, Goodlett, DR, Eng, JK, Akira, S, Underhill, DM, Aderem, A: The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature,* 410**:** 1099-1103, 2001.
42. Hagberg, L, Hull, R, Hull, S, McGhee, JR, Michalek, SM, Svanborg Eden, C: Difference in susceptibility to gram-negative urinary tract infection between C3H/HeJ and C3H/HeN mice. *Infection and immunity,* 46**:** 839-844, 1984.
43. Song, J, Abraham, SN: TLR-mediated immune responses in the urinary tract. *Current opinion in microbiology,* 11**:** 66-73, 2008.
44. Zhang, D, Zhang, G, Hayden, MS, Greenblatt, MB, Bussey, C, Flavell, RA, Ghosh, S: A toll-like receptor that prevents infection by uropathogenic bacteria. *Science (New York, NY),* 303**:** 1522-1526, 2004.
45. Chamaillard, M, Hashimoto, M, Horie, Y, Masumoto, J, Qiu, S, Saab, L, Ogura, Y, Kawasaki, A, Fukase, K, Kusumoto, S, Valvano, MA, Foster, SJ, Mak, TW, Nunez, G, Inohara, N: An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. *Nature immunology,* 4**:** 702-707, 2003.
46. Girardin, SE, Boneca, IG, Carneiro, LA, Antignac, A, Jehanno, M, Viala, J, Tedin, K, Taha, MK, Labigne, A, Zahringer, U, Coyle, AJ, DiStefano, PS, Bertin, J, Sansonetti, PJ, Philpott, DJ: Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. *Science (New York, NY),* 300**:** 1584-1587, 2003.
47. Girardin, SE, Boneca, IG, Viala, J, Chamaillard, M, Labigne, A, Thomas, G, Philpott, DJ, Sansonetti, PJ: Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *The Journal of biological chemistry,* 278**:** 8869-8872, 2003.
48. Inohara, N, Ogura, Y, Fontalba, A, Gutierrez, O, Pons, F, Crespo, J, Fukase, K, Inamura, S, Kusumoto, S, Hashimoto, M, Foster, SJ, Moran, AP, Fernandez-Luna, JL, Nunez, G: Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. *The Journal of biological chemistry,* 278**:** 5509-5512, 2003.
49. Inohara, N, Koseki, T, del Peso, L, Hu, Y, Yee, C, Chen, S, Carrio, R, Merino, J, Liu, D, Ni, J, Nunez, G: Nod1, an Apaf-1-like activator of caspase-9 and nuclear factor-kappaB. *The Journal of biological chemistry,* 274**:** 14560-14567, 1999.
50. Iwanaga, Y, Davey, MP, Martin, TM, Planck, SR, DePriest, ML, Baugh, MM, Suing, CM, Rosenbaum, JT: Cloning, sequencing and expression analysis of the mouse NOD2/CARD15 gene. *Inflammation research : official journal of the European Histamine Research Society [et al],* 52**:** 272-276, 2003.
51. Shigeoka, AA, Kambo, A, Mathison, JC, King, AJ, Hall, WF, da Silva Correia, J, Ulevitch, RJ, McKay, DB: Nod1 and nod2 are expressed in human and murine renal tubular epithelial cells and participate in renal ischemia reperfusion injury. *Journal of immunology (Baltimore, Md : 1950),* 184**:** 2297-2304, 2010.
52. Stroo, I, Butter, LM, Claessen, N, Teske, GJ, Rubino, SJ, Girardin, SE, Florquin, S, Leemans, JC: Phenotyping of Nod1/2 double deficient mice and characterization of Nod1/2 in systemic inflammation and associated renal disease. *Biology open,* 1**:** 1239-1247, 2012.
53. Halloran, PF: Immunosuppressive drugs for kidney transplantation. *The New England journal of medicine,* 351**:** 2715-2729, 2004.
54. Chan, M, Pearson, GJ: New advances in antirejection therapy. *Current opinion in cardiology,* 22**:** 117-122, 2007.
55. Faulds, D, Goa, KL, Benfield, P: Cyclosporin. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic use in immunoregulatory disorders. *Drugs,* 45**:** 953-1040, 1993.

56. Peters, DH, Fitton, A, Plosker, GL, Faulds, D: Tacrolimus. A review of its pharmacology, and therapeutic potential in hepatic and renal transplantation. *Drugs,* 46**:** 746-794, 1993.
57. Thomson, AW, Bonham, CA, Zeevi, A: Mode of action of tacrolimus (FK506): molecular and cellular mechanisms. *Therapeutic drug monitoring,* 17**:** 584-591, 1995.
58. Martinez-Martinez, S, Redondo, JM: Inhibitors of the calcineurin/NFAT pathway. *Current medicinal chemistry,* 11**:** 997-1007, 2004.
59. Kang, YJ, Kusler, B, Otsuka, M, Hughes, M, Suzuki, N, Suzuki, S, Yeh, WC, Akira, S, Han, J, Jones, PP: Calcineurin negatively regulates TLR-mediated activation pathways. *Journal of immunology (Baltimore, Md : 1950),* 179**:** 4598-4607, 2007.
60. Gonzalez-Guerrero, C, Ocana-Salceda, C, Berzal, S, Carrasco, S, Fernandez-Fernandez, B, Cannata-Ortiz, P, Egido, J, Ortiz, A, Ramos, AM: Calcineurin inhibitors recruit protein kinases JAK2 and JNK, TLR signaling and the UPR to activate NF-kappaB-mediated inflammatory responses in kidney tubular cells. *Toxicology and applied pharmacology,* 272**:** 825-841, 2013.
61. Greenblatt, MB, Aliprantis, A, Hu, B, Glimcher, LH: Calcineurin regulates innate antifungal immunity in neutrophils. *The Journal of experimental medicine,* 207**:** 923-931, 2010.
62. Herbst, S, Shah, A, Mazon Moya, M, Marzola, V, Jensen, B, Reed, A, Birrell, MA, Saijo, S, Mostowy, S, Shaunak, S, Armstrong-James, D: Phagocytosis-dependent activation of a TLR9-BTK-calcineurin-NFAT pathway co-ordinates innate immunity to Aspergillus fumigatus. *EMBO molecular medicine,* 7**:** 240-258, 2015.
63. Howell, J, Sawhney, R, Testro, A, Skinner, N, Gow, P, Angus, P, Ratnam, D, Visvanathan, K: Cyclosporine and tacrolimus have inhibitory effects on toll-like receptor signaling after liver transplantation. *Liver transplantation : official publication of the American Association for the Study of Liver Diseases and the International Liver Transplantation Society,* 19**:** 1099-1107, 2013.
64. Jennings, C, Kusler, B, Jones, PP: Calcineurin inactivation leads to decreased responsiveness to LPS in macrophages and dendritic cells and protects against LPS-induced toxicity in vivo. *Innate immunity,* 15**:** 109-120, 2009.
65. Rodrigues-Diez, R, Gonzalez-Guerrero, C, Ocana-Salceda, C, Rodrigues-Diez, RR, Egido, J, Ortiz, A, Ruiz-Ortega, M, Ramos, AM: Calcineurin inhibitors cyclosporine A and tacrolimus induce vascular inflammation and endothelial activation through TLR4 signaling. *Scientific reports,* 6**:** 27915, 2016.

Chapter 2

Calcineurin inhibitor Tacrolimus impairs host immune response against urinary tract infection

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Abstract

Calcineurin inhibitor Tacrolimus, is a potent immunosuppressive drug widely used in order to prevent acute graft rejection. Urinary tract infection (UTI) is the most frequent infectious complication in renal transplant patients and long-term use of Tacrolimus might be involved in higher susceptibility to bacterial infections. It remains largely unknown how Tacrolimus affects the host innate immune response against lower and upper UTI. To address this issue, we used experimental UTI model by intravesical inoculation of uropathogenic *E.coli* in female wild-type mice pre-treated with Tacrolimus or solvent (CTR). We found that Tacrolimus pre-treated mice displayed higher bacterial loads (cystitis, pyelonephritis and bacteremia) than CTR mice. Granulocytes from Tacrolimus pre-treated mice phagocytized less *E. coli*, released less MPO and expressed decreased levels of CXCR2 receptor upon infection. Moreover, Tacrolimus reduced TLR5 expression in bladder macrophages during UTI. This immunosuppressive state can be explained by the upregulation of TLR-signaling negative regulators (A20, ATF3, IRAK-M and SOCS1) and parallel downregulation of TLR5 as observed in Tacrolimus treated granulocytes and macrophages. We conclude that Tacrolimus impairs host innate immune responses against UTI.

Introduction

Urinary tract infection (UTI) is the most common bacterial infection in renal transplant patients[1]. The incidence of UTIs after kidney transplantation ranges from 6% to 86%[2,3]. UTIs leading to acute pyelonephritis can contribute to graft loss and mortality[4]. In the majority of cases, *E. coli* is the causative agent of UTIs[5]. The standard use of trimethoprim-sulfamethoxazole prophylaxis within the first 6 month after renal transplantation seems to be ineffective to prevent the high incidence and harm of UTIs[6]. One of the risk factors for developing UTI after renal transplantation is the lifelong use of immunosuppression in order to prevent acute graft rejection[7]. Calcineurin inhibitor Tacrolimus with its potent inhibitory effects on adaptive immunity, is one of the predominant anti-rejection agents used nowadays[8].

A proper innate immune response is of great importance in clearing bacterial infections and mainly dependent on pathogen recognition by pattern recognition receptors (PRR) such as Toll-like receptors (TLRs)[9-11]. Previous studies have shown that TLR4, TLR5 and TLR11 play a crucial role in the immune reaction against UTIs[12-14]. Activation of the NF-κB pathway via TLRs in tissue resident leukocytes with invading *E. coli* leads to a robust inflammatory response[10]. Subsequently, released chemokines attract granulocytes from circulation to the infected tissues via the CXCR2 chemokine receptor[15]. Granulocytes control the infection by various mechanisms including the ability to produce oxidative burst and phagocytosis of pathogens[16]. This teamwork between tissue resident myeloid cells and circulating granulocytes is required to properly clear UTIs[9,17].

Calcineurin is a negative regulator of the TLR-mediated activation pathway by interacting with MyD88, TRIF, TLR2 and TLR4[18]. It has been shown that calcineurin inhibition by Tacrolimus decreases responsiveness to LPS in macrophages and protects against LPS-induced toxicity in mice[19]. In human studies, it has been implicated that both calcineurin inhibitors Tacrolimus and Cyclosporine A have inhibitory effects on TLR signaling of myeloid cells post liver transplantation[20]. Moreover, a study showed that Cyclosporine A impairs Nucleotide-Binding Oligomerization Domain-Containing Protein 1 (NOD1)-mediated innate antibacterial renal defenses in mice and transplanted patients[21]. Despite the known interaction between calcineurin inhibitors and TLR pathway, it remains largely unknown how Tacrolimus may affect host antimicrobial defense mechanism against

UTIs. Therefore, the aim of this study was to investigate if and how Tacrolimus suppresses the innate immune response against lower and upper UTI.

Results

Tacrolimus enhances the susceptibility to cystitis, pyelonephritis and bacteremia

To investigate if Tacrolimus impairs host immune defense against lower and upper UTI, we induced UTI by intravesical inoculation with *E. coli* strain 1677 with solvent or Tacrolimus pre-treated mice and subsequently examined bacterial outgrowth in bladder and kidney homogenates 24 and 48h later. At both time points, Tacrolimus pre-treated mice displayed higher amount of *E. coli* colony forming units (CFUs) in both organs (Figure 1A, B). Blood cultures remained negative in all control mice, while 27% of Tacrolimus pre-treated mice showed positive blood cultures after 24h of infection (Figure 1C). These data demonstrate that Tacrolimus increases susceptibility to cystitis, pyelonephritis and bacteremia.

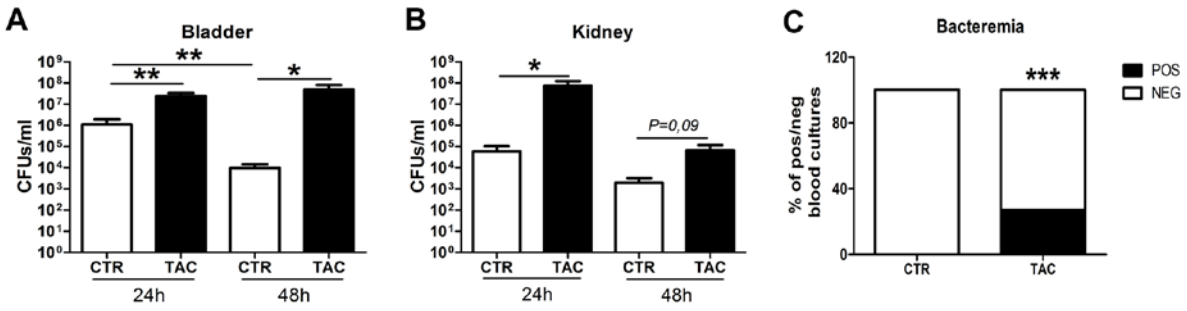

Figure 1: Higher bacterial load in Tacrolimus pre-treated mice during urinary tract infection. Bacterial load was quantified by determining colony forming units/ml in total bladder (A) and kidney (B) homogenates after 24 and 48h of infection in solvent (CTR) and Tacrolimus (TAC) pre-treated mice. Percentage of bacteremia after 24h (C). Data are expressed as mean ± SEM in A and B. **P*≤0.05, ***P*<0.01 and ****P*<0.0001. N=8/group.

Tacrolimus decreases primary functions of granulocytes

Granulocytes are the first cells that are recruited to the bladder in the early hours of UTI thereby playing a major role in bacterial clearance[16]. Granulocytes control the infection via several mechanisms, including the upregulation of CXCR2 chemokine receptor, which enables their migration towards the infected tissue. Upon entry into urinary tract, granulocytes release MPO and phagocytose *E. coli*[15,16]. Granulocyte influx is closely related to bacterial burden during UTI, in other words; more CFUs leading to more granulocyte recruitment[15,16].

Since Tacrolimus pre-treatment resulted in higher bacterial burden, we wanted to test if Tacrolimus would affect the antimicrobial properties of granulocytes. We found that blood granulocytes from Tacrolimus pre-treated mice were less efficient in phagocytizing *E. coli ex vivo*, as reflected by the decreased percentage of *E. coli* positive granulocytes and lower MFI value compared to controls (Figure 2A-C). Upon infection, percentage of circulating granulocytes and expression of CXCR2 on granulocytes were significantly reduced by Tacrolimus (Figure 2D, E). BM-granulocytes from Tacrolimus pre-treated mice released moreover significantly less MPO in response to *E. coli* stimulation (Figure 2F). Overall, granulocytes from Tacrolimus pre-treated mice have impaired antimicrobial properties.

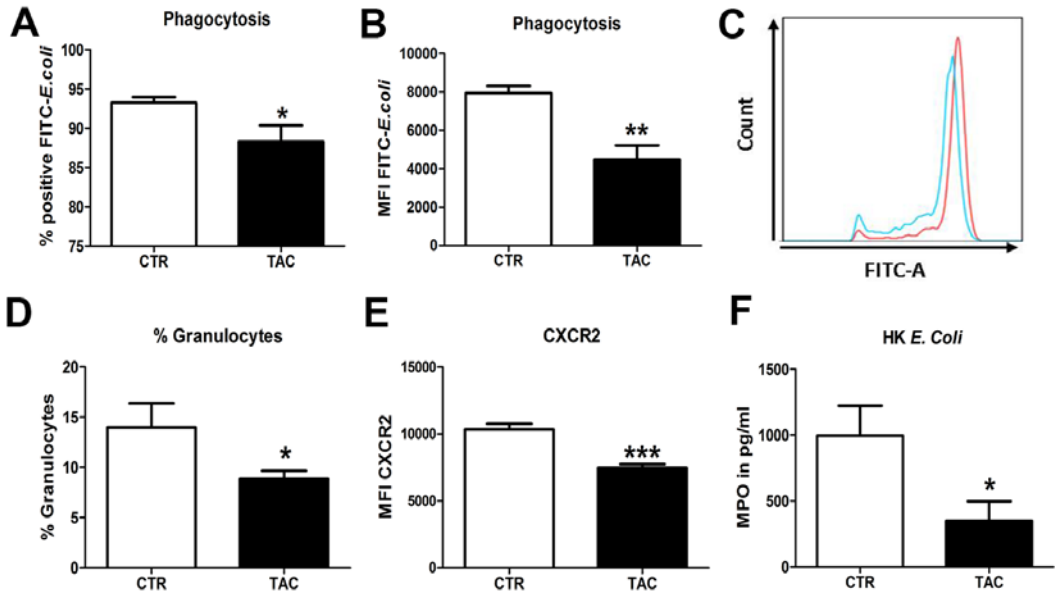

Figure 2: Tacrolimus suppresses primary functions of granulocytes. Percentage (A) and MFI (B) of FITC-labelled *E. coli* positive granulocytes from Control (CTR) and Tacrolimus (TAC) pre-treated mice. A representative FACS plot of FITC-positive granulocytes from Control (red) and Tacrolimus (blue) treated mice (C). Percentage of circulating granulocytes after 3h of infection in Control and Tacrolimus pre-treated mice (D). MFI of CXCR2 on blood granulocytes after 3h of infection in Control and Tacrolimus pre-treated mice (E). MPO release in response to HK *E. coli* by BM-granulocytes isolated from Control and Tacrolimus pre-treated mice (F). Data are expressed as mean ± SEM. **P*≤0.05, ***P*<0.01 and ****P*<0.01. N=5/8 per group.

Tacrolimus decreases TLR5 expression in bladder macrophages during urinary tract infection

Prior to granulocyte infiltration, invading *E. coli* is recognized by several TLRs including TLR4, TLR5 and TLR11[12-14]. TLR4 and TLR5 are expressed in kidney and bladder cells while TLR11 is only expressed in the kidney[10,12-14]. Gene expression analysis of these TLRs in kidney and bladder homogenates revealed that only TLR5 was reduced in the bladder of Tacrolimus pre-treated mice upon infection (supplementary figure 1). To examine in which bladder cells TLR5 expression was reduced, we performed flow cytometry analysis of the bladder tissue. This revealed that the percentage of TLR5+ cells and the MFI of TLR5 was reduced in the CD11b+ and F4/80+ cells but not in the CD11c+ population (Figure 3A-E) and non-immune cells (supplementary figure 2). The percentage of TLR5+ monocytes in the circulation was moreover significantly lower in the Tacrolimus group, whereas in granulocytes there was only a decreased trend (*P=0,09*) in TLR5 positivity (Figure 3F). The MFI of TLR5 in these cells remained steady (Figure 3G). In conclusion, Tacrolimus reduces TLR5 expression mainly in the bladder macrophages during UTI.

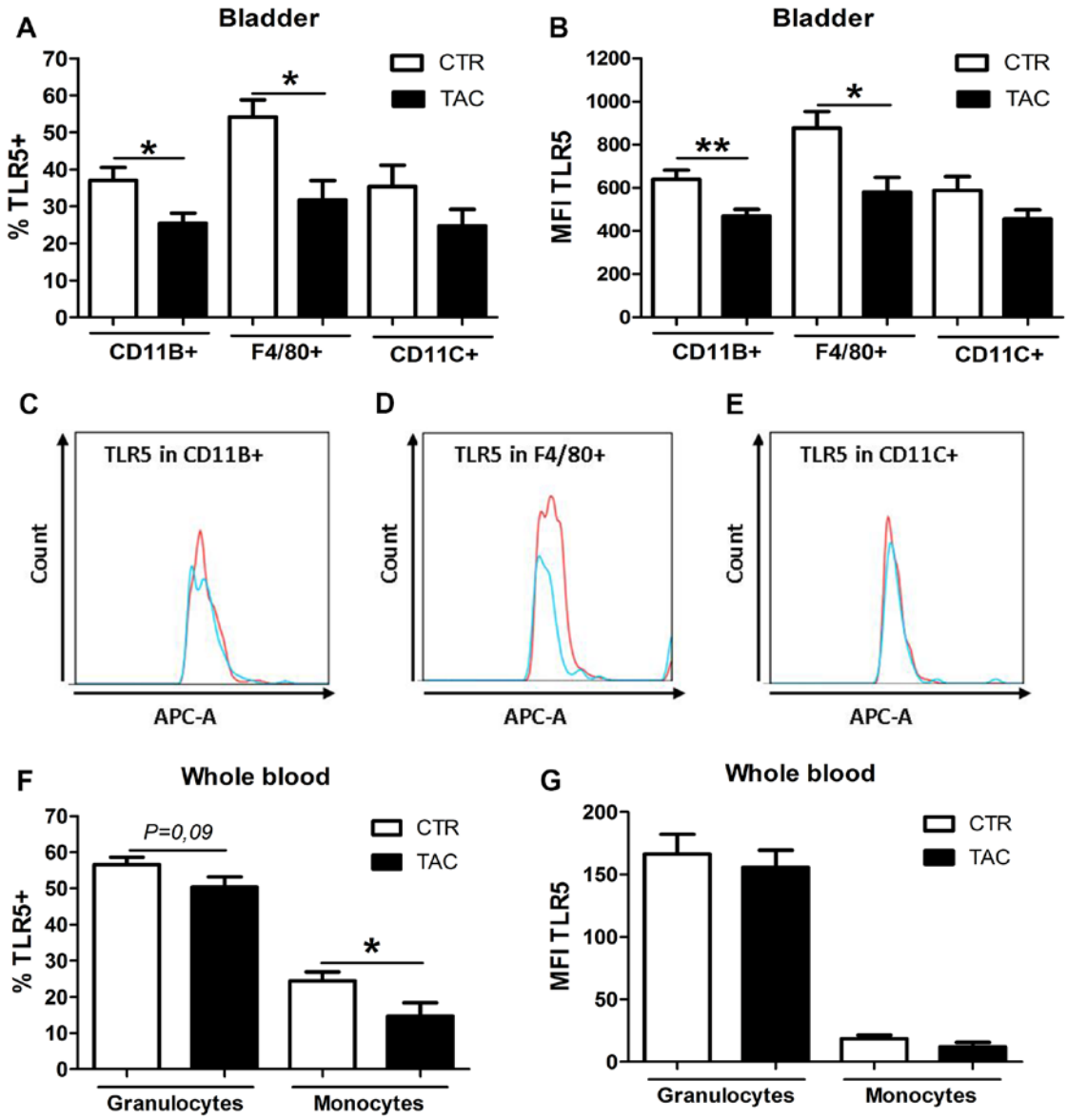

Figure 3: TLR5 expression is decreased in CD11b+ and F4/80+ bladder cells in Tacrolimus pre-treated mice during urinary tract infection. Percentage (A) and MFI (B) of TLR5 in CD11B+, F4/80+ and CD11C+ bladder populations with its FACS plots (C-E) after 20h of infection in solvent (CTR) and Tacrolimus (TAC) pre-treated mice. Percentage (F) and MFI (G) of TLR5 in blood granulocytes and monocytes after 20h of infection in Control (CTR) and Tacrolimus (TAC) pre-treated mice. Data are expressed as mean ± SEM. **P*≤0.05 and ***P*<0.01. N=8 per group.

Tacrolimus interferes with TLR-pathway via inhibition of TLR-negative regulator calcineurin

To understand how Tacrolimus induces an immunosuppressive state in myeloid cells, we next investigated the relation between calcineurin and TLR signaling. Calcineurin is a negative regulator of the TLR-mediated activation pathway by inhibiting the adaptor proteins MyD88 and TRIF[18]. Therefore, we treated macrophages either with solvent, LPS (positive control) or Tacrolimus and determined activation of TLR-mediated pathway. Both stimulations with LPS or Tacrolimus lead to phosphorylation of IκB-α (Figure 4A) and nuclear translocation of the NF-κB into the nucleus as assessed by western blot and immunofluorescent staining, respectively (Figure 4B-C). In line with these results, TNF-α mRNA was upregulated after LPS and Tacrolimus stimulation in macrophages and granulocytes (Figure 4D). Thus, Tacrolimus alone activate NF-κB pathway and hence may influence TLR pathway in myeloid cells.

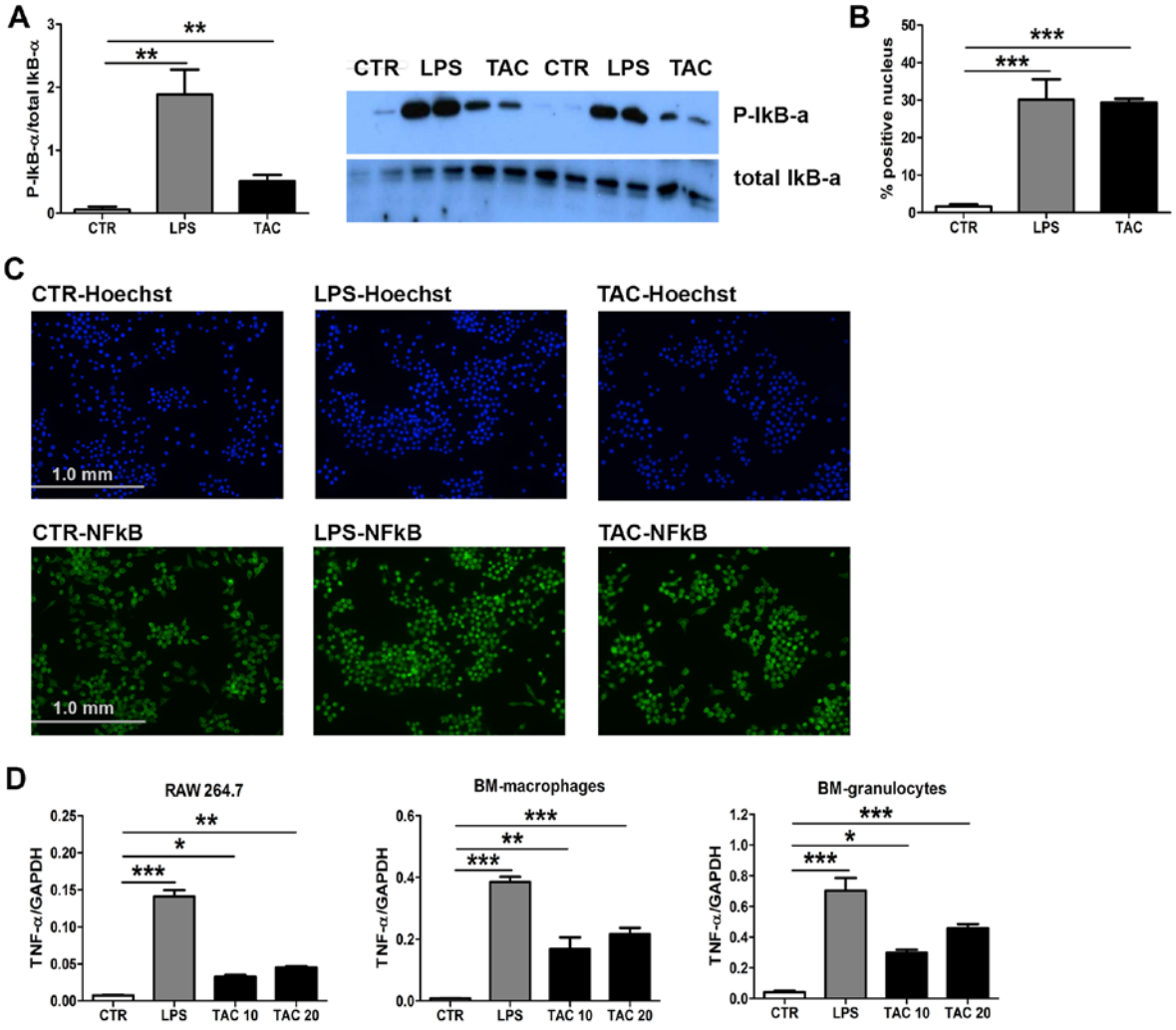

Figure 4: Tacrolimus induces activation of the classical NF-κB pathway. Protein expression of phospho-IκB-α corrected for total IκB-α (same gel) in RAW 264.7 macrophages stimulated with solvent (CTR), 100µg/ml LPS or 20µg/ml Tacrolimus (TAC) for 1h (A), full-length blots are presented in supplementary figure 3. Percentage of NF-κB positive nuclei (B). Representative stainings of nuclear translocation of NF-κB in RAW 264.7 macrophages stimulated with solvent, 100µg/ml LPS or 20µg/ml Tacrolimus for 1h (C). mRNA expression of TNF-α in RAW 264.7 macrophages, BM-macrophages and BM-granulocytes after stimulation with either solvent, 100µg/ml LPS or 10 and 20µg/ml Tacrolimus for 4h (D). Data are expressed as mean ± SEM. **P*≤0.05, ***P*<0.01 and ****P*<0.01. N=4 per group.

Tacrolimus upregulates TLR negative regulators in macrophages and granulocytes

LPS-induced NF-κB activation in myeloid cells eventually leads to endotoxin tolerance through upregulation of negative regulators of TLR-signaling that prevent excessive inflammatory reactions[22,23]. Since Tacrolimus activates NF-κB, we hypothesized that stimulation of myeloid cells with Tacrolimus might also result in the induction of negative

regulators similarly to LPS stimulation. In line with our hypothesis, we found that the main NF-κB negative regulators; A20, ATF3, IRAK-M and SOCS1 were all increased in a macrophage cell line (Figure 5A), Bone marrow (BM)-macrophages (Figure 5B) and BM-granulocytes (Figure 5C) after Tacrolimus stimulation as after LPS stimulation.

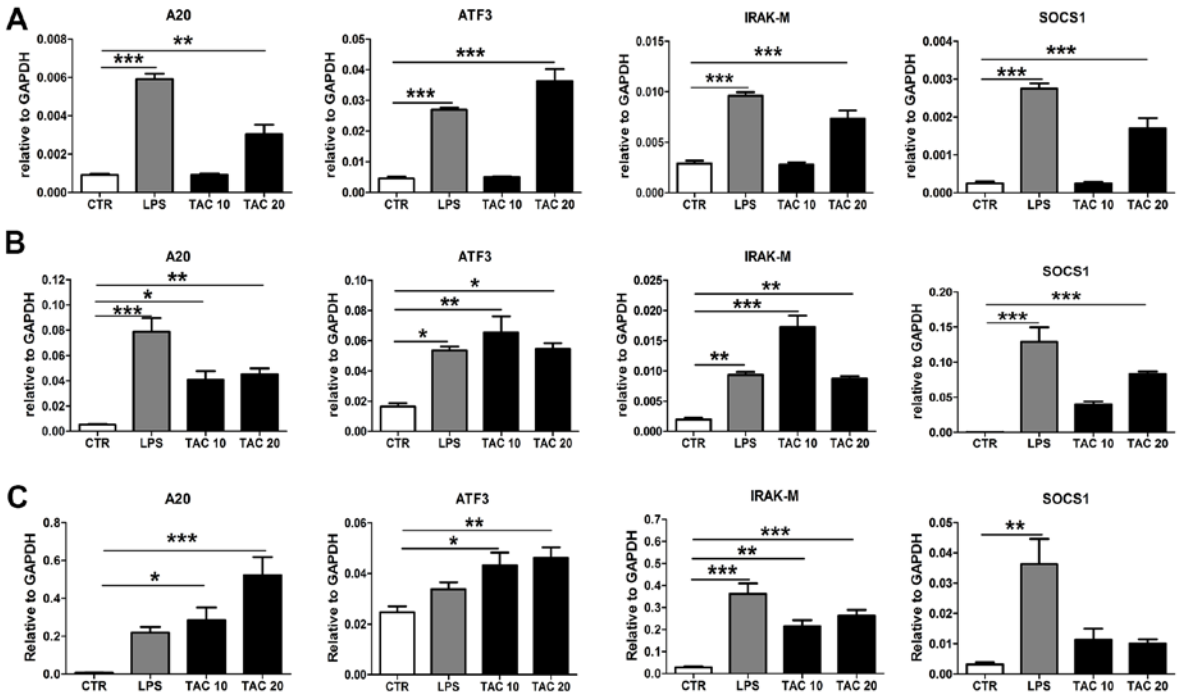

Figure 5: Tacrolimus induces upregulation of TLR negative regulators. mRNA expression of A20, ATF3, IRAK-M and SOCS1 in RAW 264.7 macrophages (A), BM-macrophages (B) and BM-granulocytes (C) after stimulation with either solvent (CTR), 100µg/ml LPS or 10 and 20µg/ml Tacrolimus (TAC) for 4h. Data are expressed as mean ± SEM. **P*≤0.05, ***P*<0.01 and ****P*<0.01. N=4 per group.

Tacrolimus induces tolerance to LPS in macrophages

To verify whether the observed increased expression of negative regulators in Tacrolimus stimulated cells would lead to endotoxin tolerance, we pre-treated macrophages either with LPS (positive control) or Tacrolimus for 24 hours after which cells were washed and re-stimulated with a second "hit" of LPS. Indeed, Tacrolimus pre-treated macrophages were significantly less responsive to LPS as reflected by the remarkably reduction of TNF-α, MIP-2 and KC release (Figure 6). Taken together, similar to endotoxin tolerance mechanism, Tacrolimus induces tolerance to LPS via induction of negative regulators in macrophages.

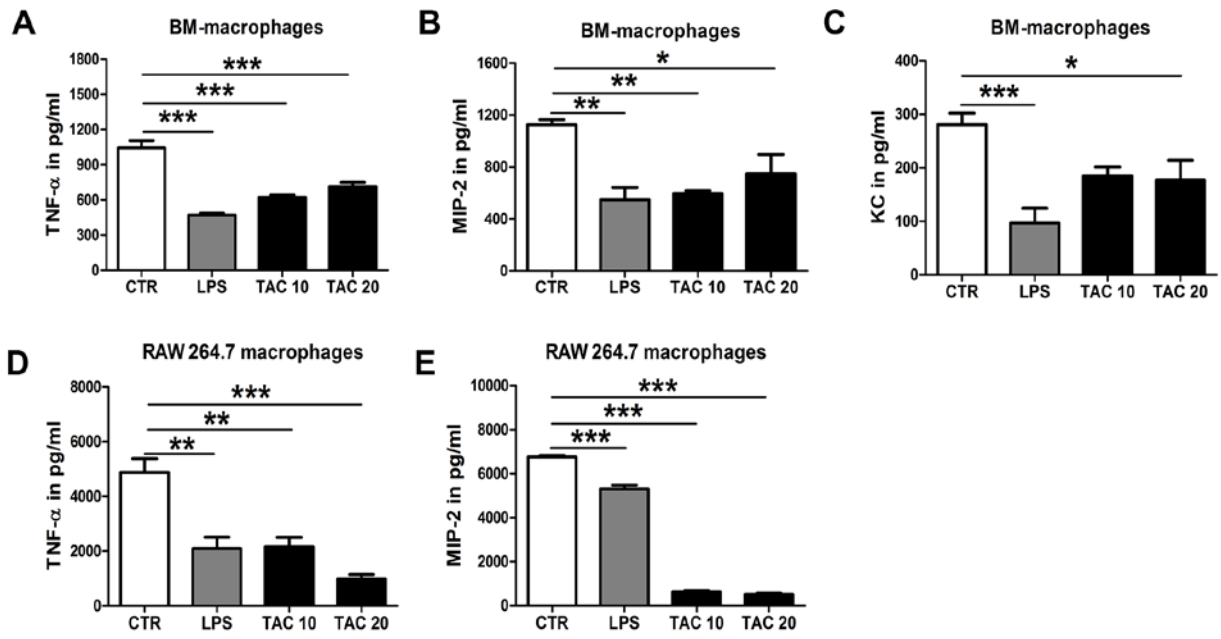

Figure 6: Tacrolimus induces a LPS-tolerant state. BM-macrophages (A) and RAW 264.7 macrophages (B) pre-treated with either solvent (CTR), 100µg/ml LPS or 10 and 20µg/ml Tacrolimus (TAC) for 24h, after washing steps cells were re-stimulated with 100ng/ml LPS for 4h. Release of TNF-α, MIP-2 and KC was measurement in cell supernatants. Data are expressed as mean ± SEM. **P*≤0.05, ***P*<0.01 and ****P*<0.01. N=4 per group.

Tacrolimus decreases TLR5 expression in granulocytes and macrophages

Next to the induction of TLR negative regulators, downregulation of TLR expression could also be a mechanism by which cells prevent an uncontrolled inflammatory response[22]. As Tacrolimus reduced the expression of TLR5 in bladder macrophages during UTI, we analyzed the expression of TLR5 and TLR4 in BM-macrophages and –granulocytes. Strikingly, we found that similarly to LPS stimulation, Tacrolimus treatment strongly decreased TLR5 expression but not TLR4 in BM-macrophages and BM-granulocytes (Figure 7A,B).

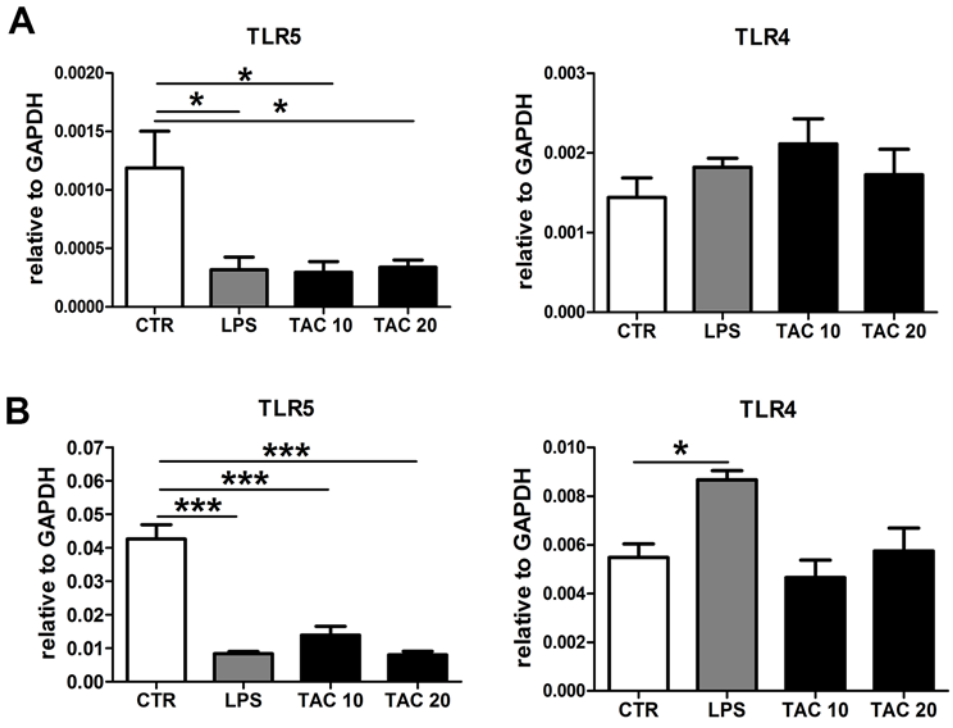

Figure 7: Tacrolimus reduces TLR5 expression during tolerant state. mRNA expression of TLR5 and TLR4 in BM-macrophages (A) and BM-granulocytes (B) after stimulation with either solvent (CTR), 100µg/ml LPS or 10 and 20µg/ml Tacrolimus (TAC) for 4h. Data are expressed as mean ± SEM. **P*≤0.05, ***P*<0.01 and ****P*<0.01. N=4 per group.

Whole blood cells of renal transplant patients respond less to LPS and heat-killed *E. coli*

Next, we determined the antimicrobial properties of myeloid cells from renal transplant patients under Tacrolimus treatment as part of their immunosuppressive drug regimens. We did not find any differences in MPO release and phagocytosis capacity of granulocytes (data not shown), meaning that the main granulocyte functions are unaffected in these patients. However, in line with our *in vitro* and *in vivo* results in mice, we found that whole blood leukocytes from Tacrolimus-receiving patients release less TNF-α and IL-8 (human equivalent for murine KC) after LPS stimulation as compared to whole blood cells from healthy individuals (Figure 8A). This trend was also seen after *E. coli* stimulation (Figure 8B). These data indicate that myeloid cells from Tacrolimus-receiving individuals are less responsive to LPS/*E. coli* stimulation.

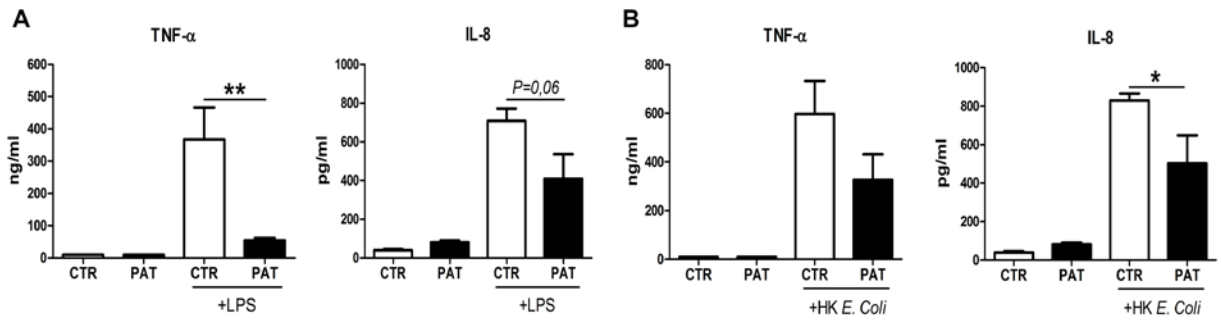

Figure 8: Leukocytes from renal transplant patients respond less to LPS and E. coli stimulus. Release of TNF-α and IL-8 after LPS (A) or *HK. E. Coli* stimulus by whole blood cells from healthy controls (CTR) and renal transplant patients (PAT). Data are expressed as mean ± SEM. *P≤0.05 and **P<0.01. N=5 per group.

Discussion

Given the high susceptibility of transplant patients to bacterial infections[1-3,7], this study aims to shed light on the potential implication of Tacrolimus treatment to the higher incidence of UTI. Present study shows that Tacrolimus impairs immune responses against UTI by inhibiting several crucial parts of the antimicrobial defense mechanism. We found that mice pre-treated with Tacrolimus had higher bacterial burden (cystitis, pyelonephritis and bacteremia) upon UTI due to impaired granulocyte and macrophage response. Firstly, granulocytes from Tacrolimus pre-treated mice were less efficient in phagocytizing *E. coli;* secondly, they produced less MPO upon stimulation with *E. coli*; lastly, the chemokine receptor CXCR2 was markedly decreased in circulating granulocytes in the early hours of infections. Moreover, TLR5 which is crucial in defense against UTIs by recognizing bacterial flagellin[24] was significantly reduced in bladder macrophages during infection. These effects appear to be driven by the induction of an immune-tolerant state by Tacrolimus. In macrophages, Tacrolimus pre-treatment lead to reduced responsiveness to LPS, as reflected by decreased release of TNF-α, KC and MIP-2. Furthermore, Tacrolimus activates the classical NF-κB pathway and induces the expression of its negative regulators A20, ATF3, IRAK-M and SOCS1 in both macrophages and granulocytes. This is likely due to the disruption of calcineurin-mediated inhibition of NF-κB signaling transduction by Tacrolimus as supported by the literature[18,19]. Taken together, our data show that the immunosuppressive state caused by Tacrolimus in macrophages and granulocytes results in higher bacterial burden during UTI.

A teamwork between granulocytes and macrophages is required for an optimal host defense against *E. coli* in the urinary tract system[17]. Resident macrophages in the bladder act like sentinels, recognizing *E. coli* via TLRs and responding to it by releasing pro-inflammatory mediators which in turn will attract granulocytes to the infected tissue. Although the role of macrophages during UTI is not extensively investigated as compared to granulocytes, two recent studies have highlighted the importance of macrophages during immune response against UTI[17,25]. Resident F4/80+ macrophages are the most populous immune cells in the naïve murine bladders[25]. Macrophages are the principal cells that acquire *E. coli* at early time points post infection and start to produce TNF-α, KC and MIP-2 in order to coordinating the exact recruitment and response of granulocytes in the infected urothelium[17,25]. Here, we show that Tacrolimus decreases macrophages response to LPS by dampening TNF-α, MIP-2 and KC production. In line with our results, another study has demonstrated that Tacrolimus inhibits macrophage inflammatory response, leading to impaired granulocyte recruitment and defective clearance of *Aspergillus fumigatus*[26]. In regard to granulocytes, our data show that the major functions of granulocytes, such as phagocytosis and MPO release are impaired by Tacrolimus. Accordingly, two independent studies showed that another calcineurin inhibitor Cyclosporine A impairs phagocytosis of granulocytes during UTI[21] and that neutrophils lacking calcineurin are defective in killing *Candida albicans*[27]. We also found that the percentage of circulating granulocytes during UTI was reduced by Tacrolimus. In line, expression of the main receptor CXCR2, which is responsible for granulocyte recruitment during bacterial infections, was reduced on granulocytes by Tacrolimus during UTI. Indeed, CXCR2 receptor KO mice have delayed granulocyte transmigration across the urothelium leading to increased susceptibility to UTI[28].

The antimicrobial immune response begins with the recognition of the pathogen by TLRs[10]. To date, TLR11, TLR4 and TLR5 have been described to play a role in the immune defense against UTIs *in vivo*[12-14,29]. TLR11 which is a pseudogene in humans, is mainly expressed by kidney cells and controls bacterial invasion of the kidney with no significant role in the bladder[14]. TLR4, instead, is expressed in both kidney and bladder[13]. TLR5 is essential in defense mechanism against UTIs and is found in both kidney and bladder[12]. The TLR11 and TLR4 expression during UTI was unchanged by Tacrolimus, whereas expression of TLR5 was specifically reduced in bladder macrophages. Feuillet *et al.* showed that the initial inflammatory response in the bladder is TLR5-dependent[30]. In support of these

findings, TLR5 polymorphism in adult women is associated with increased risk to UTIs but not pyelonephritis[31], most presumably because TLR5 does not play a role in the kidney.

TLR-activation must be tightly regulated to prevent an exacerbated inflammatory response. The main mechanisms by which myeloid cells regulate TLR-mediated signaling is either via reducing TLR expression or via induction of negative regulators[22,23]. As Tacrolimus inhibits calcineurin, which in turn is an inhibitor of the MyD88 and TRIF-pathways[18]. We hypothesized that inhibition of a TLR-pathway inhibitor would lead to activation of NF-κB and, consequently, to induction of its negative regulators in myeloid cells. We found that several NF-κB negative regulators (IRAK-M, A20, ATF3 and SOCS1) were upregulated after Tacrolimus stimulation in macrophages and granulocytes, thereby triggering a tolerant state in which myeloid cells become less responsive to LPS or *E. coli*. Indeed, Tacrolimus decreased the secretion of TNF-α, MIP-2 and KC by macrophages in response to a secondary LPS stimulation and attenuated granulocytes responsiveness to *E. coli*. Nonetheless, the LPS receptor TLR4 expression was unchanged by Tacrolimus and also during endotoxin tolerance[32]. Although the role of TLR5 during endotoxin tolerance is still unknown, our data show that TLR5 expression is significantly decreased in macrophages and granulocytes activated by Tacrolimus. Since this also happens after LPS stimulation and contemporary to the upregulation of NF-κB negative regulators, it might be argued that the downregulation of TLR5 is a characteristic of endotoxin tolerance. To summarize, our data show that Tacrolimus, similarly to endotoxin tolerance-phenomena, induces tolerance in myeloid cells. This immunosuppressive state occurs by fading the calcineurin-mediated NF-κB signaling inhibition and subsequently by inducing the NF-κB-dependent induction of its negative regulators which in turn reduce further response to endotoxin. Endotoxin tolerance is related to impaired resistance against several infectious complications[33,34]. Presumably, the immunotolerant state caused by Tacrolimus inhibits the immune response against UTI in the same manner.

It must be mentioned that concentrations of Tacrolimus used in our experimental setup should not be compared to concentrations achieved in patients. Drug pharmacokinetics and pharmacodynamics differ a lot among species and therefore this difference should be taken under consideration during designing of an experiment[35]. For instance, mice can handle much higher drug concentrations compared to humans. The concentration of 5mg/kg/day *in vivo* is a widely used dose of Tacrolimus in mouse models[26,36]. This particular dose has even been

shown to be most effective in nerve transplantation in mice[36]. Nevertheless, during the whole *in vivo* experiment, mice were critically observed for signs of discomfort, by checking the body weight and behavior. Weight loss in mice is a strong indicator for toxicity during drug treatment. In our study, after 3 days of Tacrolimus treatment, mice did not lose weight compared to solvent-treated mice as shown in supplementary figure 4, which indicates that Tacrolimus dose was not toxic. In addition, these mice were behaving normal (no signs of decreased mobility).

Papers indicating an increased incidence of UTIs in renal transplant patients do not directly support a role of immunosuppression, not because they did not find an association but because they did not look at immunosuppressive regimen as a varying variable. Early UTIs (< 6 month post transplantation) can indeed be explained in many cases by surgical procedure and anatomical position of the donor kidney. However, late UTIs are also common and not benign and this is most likely explained by the long-term use of immunosuppression, including Tacrolimus[4]. Therefore, all patients included in our study were > 6 month post-transplantation. To support our hypotheses, high incidences of UTI in non-renal transplant patients is not uncommon (liver, heart and lung transplantation) with *E. coli* being the most common causative pathogen[37], which indicates that non-anatomical, hence immunosuppression is related to high incidence of UTIs as well[38]. It has been revealed that in patients, Cyclosporine A and Tacrolimus regimes have inhibitory effects on TLR signaling post liver transplantations[20]. PBMCs from patients using Tacrolimus respond significantly less to TLR4-mediated TNF-α release[20]. Comparably, we found that leukocytes from whole blood of renal transplant patients receiving Tacrolimus have diminished TLR4-induced TNF-α release. However, we did not find any changes in the phagocytosis capacity and MPO release in granulocytes from the same patients. A previous study has shown that phagocytosis of *E. coli* was reduced in renal transplant patients, however these patients were being administered Cyclosporine A and not Tacrolimus[21]. It must be stressed that investigating innate immune cells from renal transplant patients is challenging because their immunosuppressive drug regimens do not include solely Tacrolimus/Cyclosporine A but a whole panel of other immunosuppressive agents as well. Renal transplant patients on only one immunosuppressive drug is very rare and therefore almost impossible to include in a study. Therefore, we have performed *in vivo* experiments with mice pre-treated either with MFF or Prednisone only, and found that the outcome of UTI was similar between CTR and drug

treated mice (data not shown). This indicates that not MFF or Prednisone but Tacrolimus might be related to higher bacterial burden during UTI in mice.

The present study is the first one showing the dramatic effects of Tacrolimus on functionality of macrophages and granulocytes leading to impaired antimicrobial defense against UTI. The knowledge from this study can be used in the future to develop immunosuppressive agents which aim to selectivity inhibit the adaptive immunity, thereby preventing graft loss, with no negative effects on the innate immunity. In this way, renal transplant patients will gain a higher quality of life in which bacterial infections will no longer be a concern.

Materials & Methods

Mice

C57Bl/6 wildtype mice were purchased from Charles River (Maastricht, The Netherlands). Mice were housed under specific pathogen-free conditions and received water and food ad libitum. All animal experiments were approved by the Animal care and Use Committee of the University of Amsterdam, confirming that all experiments were performed in accordance with relevant guidelines and regulations.

Tacrolimus treatment

8-12 weeks old female mice were pre-treated daily with an i.p. injection of 5mg/kg Tacrolimus (LC laboratories) or solvent (olive oil) for 3 consecutive days. For *in vitro* experiments cells were incubated with 10 or 20µg/ml of Tacrolimus, a concentration range that has been used previously[19,39,40]. Viability of cells treated with Tacrolimus was determined with MTT assay (Sigma Aldrich) (supplementary figure 5). Tacrolimus was tested for endotoxin contamination with Toxin Sensor Gel Clot Endotoxin assay kit (GenScript) according to manufacturer's instructions.

Urinary tract infection

UTI was induced as described previously with small modification of the protocol[41-43]. Briefly, *E. coli* strain 1677, isolated from an uroseptic patient was grown overnight at 37^{o}C in

Tryptone Soy Broth (TSB) medium, followed by 1:100 dilution in TSB medium. After reaching an optical density of 1 (OD_{600nm}), *E. coli* was washed twice in ice-cold PBS and resuspended in 5ml of PBS. Infection was induced by transurethral inoculation of 50µl of *E. coli* suspension into the bladder by 0.55µm catheter (Abbott). UTI was induced under general anesthesia, induction rate was 3% of Isoflurane and 1-2% for maintenance. After inoculation of *E. coli* into the bladder, mice were kept under anesthesia for 1 hour. Subsequently, under general anesthesia, mice were sacrificed after 3, 24 and 48 hours of infection by heart puncture followed by cervical dislocation.

Bacterial outgrowth

Bacterial outgrowth was determined as described previously[41]. Briefly, bladder and the left kidney were homogenized in PBS with a tissue homogenizer. The homogenizer was cleaned twice with 70% alcohol and once with PBS after each sample. Tissue homogenates were diluted in serial 10-fold dilutions in PBS, 50µl of homogenates and blood were plated out onto blood agar plates at 37°C o/n. The next day, *E. coli* colony forming units (CFUs) were counted.

ELISA

Cytokine, chemokine and MPO levels were measured in cell supernatants or bladder and kidney homogenates with specific ELISA DuoSet kits (R&D Systems) according to manufacturer's instructions. The total amount of protein in tissue homogenates was measured with BCA assay.

BM-granulocytes, BM-macrophages and RAW 264.7 macrophages

BM-granulocytes and BM-macrophages were isolated/generated as described previously[44]. Briefly, femoral and tibial bone marrow was flushed with ice-cold sterile PBS with a 21-gauge needle above a 40µm pore filter. Granulocytes were isolated by means of magnetic labeling using anti-Ly6G Microbead Kit, according to manufacturer's instructions (Miltenyi Biotec). BM-macrophages were generated by culturing total bone marrow cells in petri dishes (Greiner) with bone marrow macrophage medium (RPMI culture medium (Gibco) supplemented with 10% FCS, 100IU/ml penicillin, 100µg/ml streptomycin, 2mM L-Glutamine (Invitrogen) and 15% of LCM (L929 conditioned medium)) for 7 days. RAW

264.7 macrophage cell line was cultured in DMEM (Gibco) culture medium containing 10% FCS, 100 IU/ml penicillin, 100µg/ml streptomycin, 2 mM L-Glutamine (Invitrogen). Cells were passaged by ~80% confluency. Macrophages were stimulated with solvent, 100ng/ml LPS, 10 or 20ug/ml of Tacrolimus for 24 hours, after which cells were washed 3 times with PBS and re-stimulated with 100ng/ml LPS for 4 hours. Supernatants were collected and stored at -20 ^{o}C until analysis. To determine expression of negative regulators on mRNA level, cells were stimulated with the same concentrations of LPS or Tacrolimus for 4 hours, followed by treatment with Tri-reagent after which samples were collected and stored at -80^{o}C for further process.

Reverse transcriptase-PCR

Kidney, bladder homogenates and cells were treated with Tri-reagent (Sigma Aldrich) to isolate total RNA according to the manufacturer's protocol. cDNA was synthesized using oligo-dt as primer. mRNA expression of several genes was measured by RT-PCR performed on a Light Cycler 480 (Roche) with SYBR green PCR master mix (Bioline). Intensity of SYBR green dye was determined by linear regression analysis (LinRegPCR, developed by Heart Failure Research Center, Amsterdam, The Netherlands). Expression of specific genes were normalized to expression of the house keeping gene GAPDH.

Phagocytosis assay

Phagocytosis of *E. coli* by blood granulocytes was measured with PHAGOTEST (Glycotope Biotechnology) according to the manufacturer's instructions. Briefly, 100µl of heparinized whole blood was stained with opsonized FITC-labelled E.coli and incubated for 10 minutes at 37^{o}C (water bath), while the negative control remained on ice. After phagocytosis was stopped, quenching solution was used to discriminate between surface bound and internalized *E. coli*. After lysing of the red blood cells and DNA staining to discriminate *E. coli* from granulocytes, phagocytic capacity was measured by flow cytometry on a FACS Canto (BD Bioscience) and analyzed with FlowJo version 10.

Western blotting

Macrophages were lysed in RIPA buffer supplemented with 4mM Na_3VO_4, 10mM NaF and 1:100 protease inhibitor (Sigma Aldrich). Protein concentrations were determined by BCA assay. Cell lysates were denatured with 2-mercaptoethanol and incubated at 95^{o}C for 8 min,

20ug protein was loaded on a 12% SDS-PAGE gel and blotted on a blot membrane (Millipore). The blot was cut in two before incubation with the antibodies. Blots for phospho-IκBα were incubated in blocking buffer (Tris buffered saline supplemented with 0.1% Tween 20 (Sigma Aldrich)) containing 5% milk or for total IκBα in blocking buffer with 5% BSA for 1 hour). Blots were incubated with primary mouse anti-phospho-IκBα (Cell Signaling) 1:1000 antibody in blocking buffer o/n. As secondary antibody, goat anti-mouse IgG1-hrp (1:1000 in blocking buffer) (Sigma Aldrich) was used. For total IκBα, rabbit anti-mouse polyclonal IκBα (1:500) (Santa Cruz) and as secondary antibody goat anti-rabbit-hrp (1:1000) (Sigma Aldrich) was used. Samples were detected with ECL+ (Amersham) using autoradiography films (GE Healthcare). Western blot band were quantified with Image J software.

Immunofluorescence staining

Macrophages were plated out at a concentration of $0.05*10^6$ cells/well on coverslips in a 24-wells plate (Greiner). After 2 days, cells were stimulated with solvent, 100ng/ml LPS or 20µg/ml Tacrolimus for 1 hour. Cells were fixed with 4% formaldehyde for 10 minutes, followed by permeabilization and blocking step with TBP (0.1% Triton X-100, 0.5% BSA in PBS) for 30 minutes. Samples were incubated with primary antibody rabbit anti-mouse NFκB (Santa Cruz) 1:500 in TBP o/n at 4ºC. After washing step, cells were incubated with the secondary antibody goat anti-rabbit-Alexa Fluor 488 (Life Technologies) at a dilution of 1:1000 in TBP for 1 hour at RT. The nuclei were stained with 0.5µg/ml Hoechst in PBS for 30 minutes at RT. Pictures were made with Leica epifluorescence microscope software. Translocation of NFκB was determined by counting total and NF-κB positive nuclei in a blinded fashion.

White blood cells

Composition of whole blood was determined with Scil Vet abc Plus+ (HORIBA Medical) in 30µl blood sample.

Flow cytometry

Bladder tissue was minced and digested with digestion buffer containing 0,34U/ml Liberase TM and 100µg/ml DNase (Sigma Aldrich) in PBS for 1 hour at 37ºC . Cells were washed with FACS buffer and passed through a 100µm cell strainer, blocked with anti-Fc Receptor

(Anti-Mouse CD16/CD32, eBioscience) and stained with antibodies anti-CD45.2-Percp Cy5.5, CD11b-FITC, F4/80-PE or CD11C-PE and TLR5-APC (Biolegend) in FACS buffer. Stainings were visualized using a BD LSRFortessa™ cell analyser (BD Bioscience); DAPI (Sigma-Aldrich) was added to the cell suspension short before measurement to discriminate dead from alive cells. Flow cytometry data were analyzed using FlowJo v10 (Ashland, OR).

Renal transplant patients

Patient selection criteria were as follow: men and women >18 years old, at least 6 month after renal transplantation, on Tacrolimus, MMF and prednisolone >3 month, clinically stable at the time of blood donation. Healthy controls were matched with age and gender of the patients. Heparinized blood was collected and *ex vivo* stimulated with 500ng/ml LPS or HK *E. coli* for 4h hours, after which the supernatants were stored at 20^{o}C for analysis of cytokines. All human subjects signed an informed consent. This study was approved by the Medical ethical committee of the AMC, confirming that all experiments were performed in accordance with relevant guidelines and regulations.

Statistical analyses

All statistical analyses were performed using Graphpad Prism version 5 software. Results are expressed as mean ± SEM. Comparisons between two groups were analysed using the two-tailed unpaired *t* test. One-way ANOVA followed by Bonferroni's multiple comparison test was used for comparison between more than 2 groups. Fisher's exact test was used for analysis of bacteremia. Values of $P \leq 0.05$ were considered to represent a statistically significant difference.

Acknowledgments

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References

1 Golebiewska, J., Debska-Slizien, A., Komarnicka, J., Samet, A. & Rutkowski, B. Urinary tract infections in renal transplant recipients. *Transplantation proceedings* **43**, 2985-2990, doi:10.1016/j.transproceed.2011.07.010 (2011).

2 Chuang, P., Parikh, C. R. & Langone, A. Urinary tract infections after renal transplantation: a retrospective review at two US transplant centers. *Clinical transplantation* **19**, 230-235, doi:10.1111/j.1399-0012.2005.00327.x (2005).

3 Rubin, R. H. Infectious disease complications of renal transplantation. *Kidney international* **44**, 221-236 (1993).

4 Abbott, K. C. *et al.* Late urinary tract infection after renal transplantation in the United States. *American journal of kidney diseases : the official journal of the National Kidney Foundation* **44**, 353-362 (2004).

5 Rice, J. C. *et al.* Renal allograft injury is associated with urinary tract infection caused by Escherichia coli bearing adherence factors. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* **6**, 2375-2383, doi:10.1111/j.1600-6143.2006.01471.x (2006).

6 Singh, R. *et al.* The impact of trimethoprim-sulfamethoxazole as Pneumocystis jiroveci pneumonia prophylaxis on the occurrence of asymptomatic bacteriuria and urinary tract infections among renal allograft recipients: a retrospective before-after study. *BMC infectious diseases* **16**, 90, doi:10.1186/s12879-016-1432-3 (2016).

7 Fishman, J. A. & Rubin, R. H. Infection in organ-transplant recipients. *The New England journal of medicine* **338**, 1741-1751, doi:10.1056/nejm199806113382407 (1998).

8 Thomson, A. W., Bonham, C. A. & Zeevi, A. Mode of action of tacrolimus (FK506): molecular and cellular mechanisms. *Therapeutic drug monitoring* **17**, 584-591 (1995).

9 Spencer, J. D., Schwaderer, A. L., Becknell, B., Watson, J. & Hains, D. S. The innate immune response during urinary tract infection and pyelonephritis. *Pediatric nephrology (Berlin, Germany)* **29**, 1139-1149, doi:10.1007/s00467-013-2513-9 (2014).

10 Song, J. & Abraham, S. N. TLR-mediated immune responses in the urinary tract. *Current opinion in microbiology* **11**, 66-73, doi:10.1016/j.mib.2007.12.001 (2008).

11 Takeda, K. & Akira, S. Toll-like receptors. *Current protocols in immunology / edited by John E. Coligan ... [et al.]* **109**, 14.12.11-10, doi:10.1002/0471142735.im1412s109 (2015).

12 Andersen-Nissen, E. *et al.* Cutting edge: Tlr5-/- mice are more susceptible to Escherichia coli urinary tract infection. *Journal of immunology (Baltimore, Md. : 1950)* **178**, 4717-4720 (2007).

13 Hagberg, L. *et al.* Difference in susceptibility to gram-negative urinary tract infection between C3H/HeJ and C3H/HeN mice. *Infection and immunity* **46**, 839-844 (1984).

14 Zhang, D. *et al.* A toll-like receptor that prevents infection by uropathogenic bacteria. *Science (New York, N.Y.)* **303**, 1522-1526, doi:10.1126/science.1094351 (2004).

15 Olszyna, D. P. *et al.* CXC chemokine receptor 2 contributes to host defense in murine urinary tract infection. *The Journal of infectious diseases* **184**, 301-307, doi:10.1086/322030 (2001).

16 Haraoka, M. *et al.* Neutrophil recruitment and resistance to urinary tract infection. *The Journal of infectious diseases* **180**, 1220-1229, doi:10.1086/315006 (1999).

17 Schiwon, M. *et al.* Crosstalk between sentinel and helper macrophages permits neutrophil migration into infected uroepithelium. *Cell* **156**, 456-468, doi:10.1016/j.cell.2014.01.006 (2014).

18 Kang, Y. J. *et al.* Calcineurin negatively regulates TLR-mediated activation pathways. *Journal of immunology (Baltimore, Md. : 1950)* **179**, 4598-4607 (2007).

19 Jennings, C., Kusler, B. & Jones, P. P. Calcineurin inactivation leads to decreased responsiveness to LPS in macrophages and dendritic cells and protects against LPS-induced toxicity in vivo. *Innate immunity* **15**, 109-120, doi:10.1177/1753425908100928 (2009).

20 Howell, J. *et al.* Cyclosporine and tacrolimus have inhibitory effects on toll-like receptor signaling after liver transplantation. *Liver transplantation : official publication of the American Association for the Study of Liver Diseases and the International Liver Transplantation Society* **19**, 1099-1107, doi:10.1002/lt.23712 (2013).

21 Tourneur, E. *et al.* Cyclosporine A impairs nucleotide binding oligomerization domain (Nod1)-mediated innate antibacterial renal defenses in mice and human transplant recipients. *PLoS pathogens* **9**, e1003152, doi:10.1371/journal.ppat.1003152 (2013).

22 Biswas, S. K. & Lopez-Collazo, E. Endotoxin tolerance: new mechanisms, molecules and clinical significance. *Trends in immunology* **30**, 475-487, doi:10.1016/j.it.2009.07.009 (2009).

23 Fan, H. & Cook, J. A. Molecular mechanisms of endotoxin tolerance. *Journal of endotoxin research* **10**, 71-84, doi:10.1179/096805104225003997 (2004).

24 Hayashi, F. *et al.* The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* **410**, 1099-1103, doi:10.1038/35074106 (2001).

25 Mora-Bau, G. *et al.* Macrophages Subvert Adaptive Immunity to Urinary Tract Infection. *PLoS pathogens* **11**, e1005044, doi:10.1371/journal.ppat.1005044 (2015).

26 Herbst, S. *et al.* Phagocytosis-dependent activation of a TLR9-BTK-calcineurin-NFAT pathway co-ordinates innate immunity to Aspergillus fumigatus. *EMBO molecular medicine* **7**, 240-258, doi:10.15252/emmm.201404556 (2015).

27 Greenblatt, M. B., Aliprantis, A., Hu, B. & Glimcher, L. H. Calcineurin regulates innate antifungal immunity in neutrophils. *The Journal of experimental medicine* **207**, 923-931, doi:10.1084/jem.20092531 (2010).

28 Frendeus, B. *et al.* Interleukin 8 receptor deficiency confers susceptibility to acute experimental pyelonephritis and may have a human counterpart. *The Journal of experimental medicine* **192**, 881-890 (2000).

29 Leemans, J. C., Kors, L., Anders, H. J. & Florquin, S. Pattern recognition receptors and the inflammasome in kidney disease. *Nature reviews. Nephrology* **10**, 398-414, doi:10.1038/nrneph.2014.91 (2014).

30 Feuillet, V. *et al.* Involvement of Toll-like receptor 5 in the recognition of flagellated bacteria. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 12487-12492, doi:10.1073/pnas.0605200103 (2006).

31 Hawn, T. R. *et al.* Toll-like receptor polymorphisms and susceptibility to urinary tract infections in adult women. *PloS one* **4**, e5990, doi:10.1371/journal.pone.0005990 (2009).

32 Matsuguchi, T., Musikacharoen, T., Ogawa, T. & Yoshikai, Y. Gene expressions of Toll-like receptor 2, but not Toll-like receptor 4, is induced by LPS and inflammatory cytokines in mouse macrophages. *Journal of immunology (Baltimore, Md. : 1950)* **165**, 5767-5772 (2000).

33 Youngner, J. S. & Stinebring, W. R. Interferon appearance stimulated by endotoxin, bacteria, or viruses in mice pre-treated with Escherichia coli endotoxin or infected with Mycobacterium tuberculosis. *Nature* **208**, 456-458 (1965).

34 Mason, C. M., Dobard, E., Summer, W. R. & Nelson, S. Intraportal lipopolysaccharide suppresses pulmonary antibacterial defense mechanisms. *The Journal of infectious diseases* **176**, 1293-1302 (1997).

35 Nair, A. B. & Jacob, S. A simple practice guide for dose conversion between animals and human. *Journal of basic and clinical pharmacy* **7**, 27-31, doi:10.4103/0976-0105.177703 (2016).

36 Udina, E., Voda, J., Gold, B. G. & Navarro, X. Comparative dose-dependence study of FK506 on transected mouse sciatic nerve repaired by allograft or xenograft. *Journal of the peripheral nervous system : JPNS* **8**, 145-154 (2003).

37 Vidal, E. *et al.* Bacterial urinary tract infection after solid organ transplantation in the RESITRA cohort. *Transplant infectious disease : an official journal of the Transplantation Society* **14**, 595-603, doi:10.1111/j.1399-3062.2012.00744.x (2012).

38 Montoya, J. G. *et al.* Infectious complications among 620 consecutive heart transplant patients at Stanford University Medical Center. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **33**, 629-640, doi:10.1086/322733 (2001).

39 Rodrigues-Diez, R. *et al.* Calcineurin inhibitors cyclosporine A and tacrolimus induce vascular inflammation and endothelial activation through TLR4 signaling. *Scientific reports* **6**, 27915, doi:10.1038/srep27915 (2016).

40 Gonzalez-Guerrero, C. *et al.* Calcineurin inhibitors recruit protein kinases JAK2 and JNK, TLR signaling and the UPR to activate NF-kappaB-mediated inflammatory responses in kidney tubular cells. *Toxicology and applied pharmacology* **272**, 825-841, doi:10.1016/j.taap.2013.08.011 (2013).

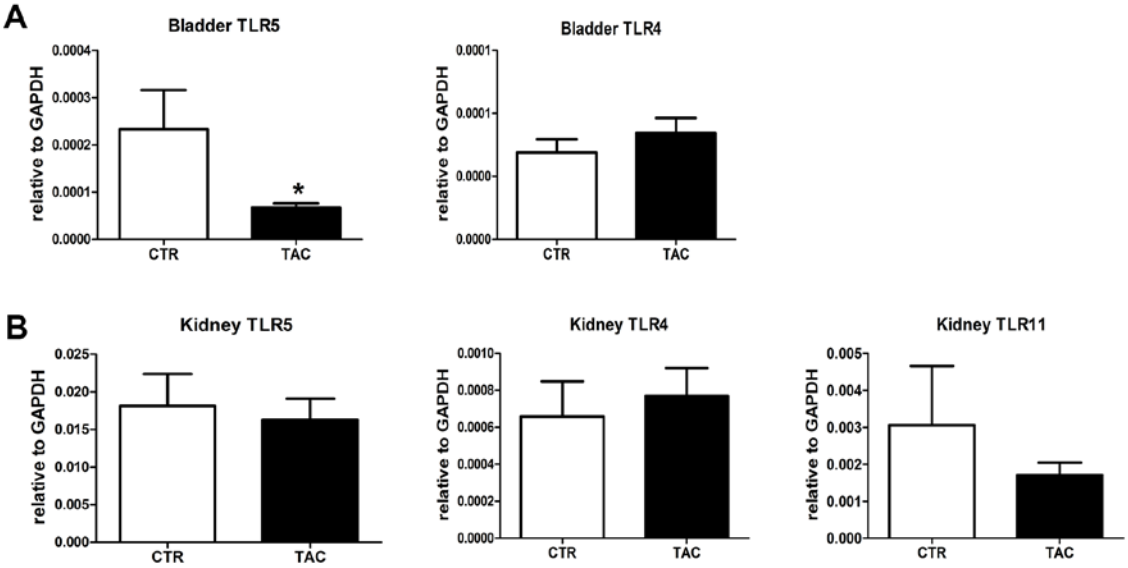
41 Dessing, M. C. *et al.* S100A8/A9 is not involved in host defense against murine urinary tract infection. *PloS one* **5**, e13394, doi:10.1371/journal.pone.0013394 (2010).

42 Roelofs, J. J. *et al.* The urokinase plasminogen activator receptor is crucially involved in host defense during acute pyelonephritis. *Kidney international* **70**, 1942-1947, doi:10.1038/sj.ki.5001947 (2006).

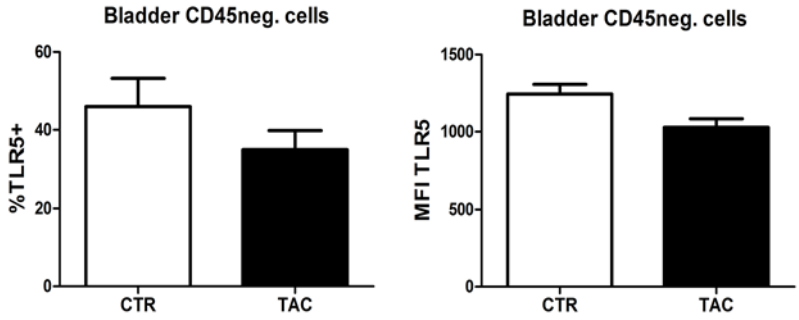
43 Lattenist, L. *et al.* The lectin like domain of thrombomodulin is involved in the defence against pyelonephritis. *Thrombosis research* **136**, 1325-1331, doi:10.1016/j.thromres.2015.11.004 (2015).

44 Emal, D. *et al.* Depletion of Gut Microbiota Protects against Renal Ischemia-Reperfusion Injury. *Journal of the American Society of Nephrology : JASN*, doi:10.1681/asn.2016030255 (2016).

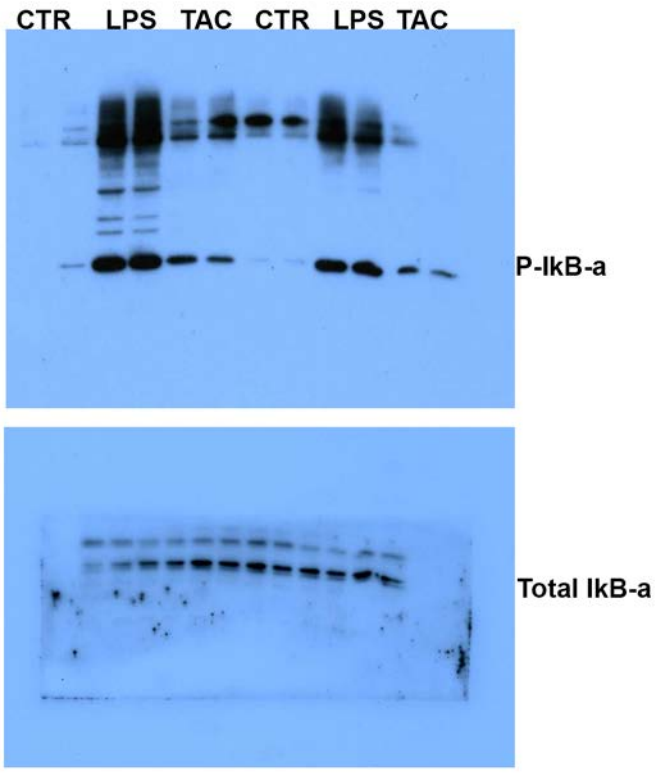
Supplementary figures



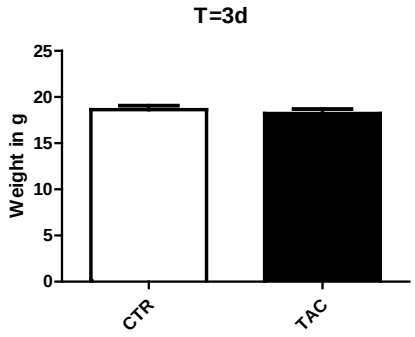
Supplementary figure 1: **Tacrolimus reduces TLR5 expression in bladder tissue during UTI**. mRNA expression of several TLRs in bladder (A) and kidney (B) tissue during UTI in solvent (CTR) and Tacrolimus (TAC) pre-treated mice. Data are expressed as mean ± SEM. N=8 per group.



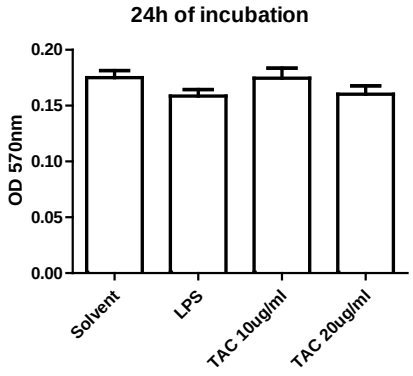
Supplementary figure 2: Expression of TLR5 in bladder non-immune cells during UTI. Percentage and MFI of TLR5 in CD45 negative bladder cell population during infection in solvent (CTR) and Tacrolimus (TAC) pre-treated mice. Data are expressed as mean ± SEM. N=8 per group.



Supplementary figure 3: Full-length blots for protein expression of Total and p-IkB-α on the same gel. Blot was cut in two before incubation with specific antibodies. N=4 per group.



Supplementary figure 4: Weight of mice after 3 days of treatment either with solvent or Tacrolimus in grams. Data are expressed as mean ± SEM. N=8 per group.



Supplementary figure 5: Viability of macrophages after 24 hours of incubation with Solvent, LPS or TAC 10-20ug/ml is comparable, as determined by MTT assay. Data are expressed as mean ± SEM. N=8 per group.

Chapter 3

Depletion of gut microbiota protects against renal ischemia/reperfusion injury

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Abstract

An accumulating body of evidence shows that gut microbiota play an important role in health and disease by modulating local and systemic immunity. The importance of the microbiome in the development of kidney disease is however largely unknown. To study this, we depleted gut microbiota with broad-spectrum antibiotics and performed renal ischemia/reperfusion (I/R)-injury in mice. Our results show that depletion of microbiota significantly attenuated renal damage, dysfunction, remote organ injury and maintained tubular integrity after renal I/R-injury. We found a reduction in expression levels of F4/80, chemokine receptors CX3CR1 and CCR2 in the F4/80+ renal resident macrophage population and bone marrow (BM)-monocytes. In addition, our data showed that BM-monocytes from gut flora-depleted mice had decreased migratory capacity towards CX3CL1 and CCL2 ligands compared to control BM-monocytes. To study whether these effects were driven by depletion of microbiota, we performed fecal transplantation in antibiotic-treated mice and found that it abolished the protective effect of microbiota depletion upon renal I/R-injury. In conclusion, we show that depletion of gut microbiota profoundly protects against renal I/R-injury by reducing maturation status of F4/80+ renal resident macrophages and BM-monocytes. Therefore, dampening the inflammatory response by targeting microbiota-derived mediators might be a promising therapy against I/R-injury.

Introduction

Acute kidney injury (AKI) is a major clinical problem affecting 5% of hospitalized patients and has a mortality rate of 50-80% in these patients[1]. Ischemia reperfusion (I/R)-injury is a major cause of AKI which can occur during shock, sepsis and renal transplantation[2]. Ischemia-induced damage leads to apoptosis and necrosis of renal tubular cells, followed by release of damage-associated molecular patterns (DAMPs) which are sensed by pathogen recognition receptors present on tubular epithelial cells (TECs) and renal resident leukocytes[3-6]. Activated TECs and resident leukocytes release cytokines/chemokines to attract inflammatory cells from circulation to the injured kidney to clear cell debris and promote repair[3, 7]. However, when the powerful inflammatory response is exaggerated it can also amplify renal damage[3, 8]. Thus, inflammation can have profound but disparate roles in the pathogenesis of AKI.

In the past few years, intestinal microbiota-related research has been rapidly expanding, providing important insights into the involvement of the microbiome in modulating systemic immunity and thereby affecting the outcome of several inflammatory diseases[9-18]. For instance, antibiotic-treated mice have impaired innate and adaptive immune responses after exposure to lung infection caused by either influenza virus or *K. Pneumonia*[9, 14, 16]. Mice lacking intestinal microbiota develop less severe symptoms in autoimmune models of arthritis and experimental autoimmune encephalomyelitis[17, 18].

In regard to renal diseases, few studies demonstrated an improved uremic state after gut microbiota-directed interventions in chronic kidney disease and end-stage renal disease patients[19-21]. Impact of the microbiome on the disease process of AKI remains however unknown, as well as a possible mechanism by which microbiota can influence the kidney. We therefore investigated the role of microbiota in I/R-injury induced AKI by depleting gut microbiota with broad-spectrum antibiotic treatment in wild type (WT) mice. We found that microbiota are essential for priming F4/80+ renal resident macrophages and BM-monocytes. Consequently, renal resident macrophages from commensal-depleted mice are less responsive to ischemic injury, resulting in protection against renal I/R-injury.

Results

Depletion of intestinal microbiota protects against renal ischemia/reperfusion injury

Following depletion of microbiota, we found a striking and profound protection against renal I/R-injury as assessed by scoring the percentage of necrotic tubules and renal expression of neutrophil gelatinase-associated lipocalin (NGAL) 24h after I/R-injury (Figure 1A-B). Renal function was significantly preserved in antibiotic-treated mice as indicated by lower plasma creatinine and urea levels compared to control mice (Figure 1C-D). LDH, ASAT, and ALAT plasma levels are known to increase upon renal I/R-injury indicating general tissue injury[22]. Conversely, in antibiotic-treated mice LDH, ASAT and ALAT levels remain steady after I/R-injury (Figure 1E-G). One day after I/R-injury the inflammatory milieu is predominantly characterized by a vast influx of granulocytes[4, 6, 7, 23], which can play a detrimental role in the post-ischemic kidney[24]. We found significant lower influx of granulocytes in antibiotic-treated mice upon I/R-injury (Figure 1H). In antibiotic-treated mice renal structural integrity was better maintained as shown by significantly less TEC apoptosis and more TEC proliferation (Figure 1I, J). Taken together, these data clearly show that depletion of microbiota protects mice against renal I/R-injury.

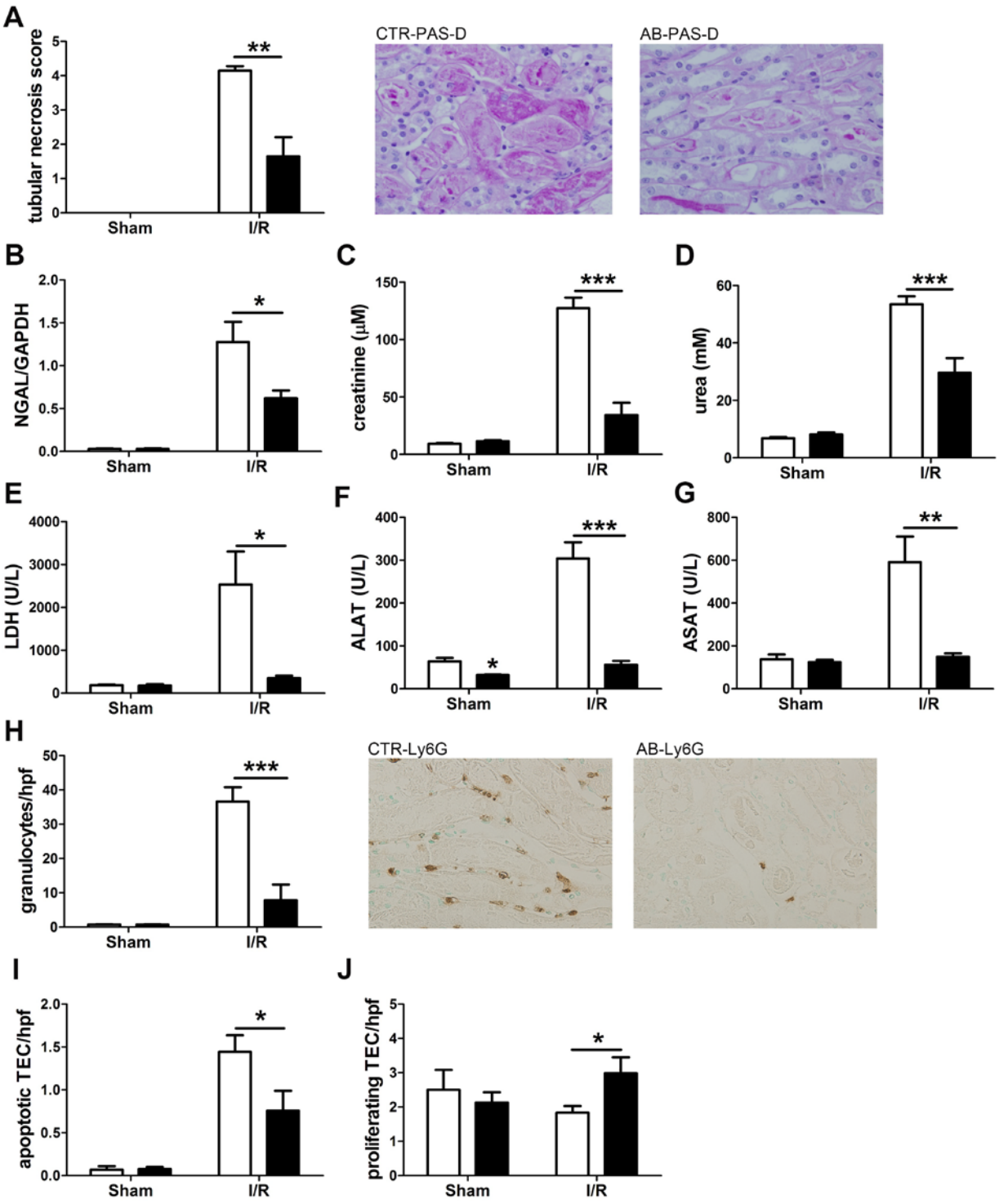

***Figure 1*: Depletion of gut microbiota protects against renal I/R-injury.** (A) Renal damage was evaluated by scoring the percentage of necrotic tubules in PAS-D-stained sections (magnification 400x) and by measuring NGAL mRNA level in total kidney homogenates from control (white bars) and antibiotic-treated mice (black bars) (B). Renal function was quantified by plasma creatinine (C) and urea (D) levels. Plasma levels of general organ damage and liver markers LDH (E), ALAT (F) and ASAT (G) were determined. Influx of granulocytes was assessed by scoring $Ly6G^+$ cells in the corticomedullary region of the kidney (H) (magnification 400x). The amount of apoptotic TEC was determined by scoring caspase-3^+ TEC (I) and the amount of proliferating TEC (J)

was determined by scoring $Ki67^+$ TEC. Data are expressed as mean ± SEM. In the I/R group, the two-tailed unpaired *t* test was used in all graphs (n=7/8 per group). **P*<0.05, ***P*<0.01 and *** *P*<0.005.

Decreased levels of renal inflammatory mediators early after ischemic damage in commensal-depleted mice

To study the mechanism underlying the absence of massive granulocyte influx into the ischemic kidneys after microbiota depletion, we next analysed the migration capacity of granulocytes and found that it was not affected by microbiota depletion (Figure S1A). In addition, the granulocyte phagocytosis and oxidative burst capacities were not altered by microbiota depletion (Figure S1B-C). Since granulocyte motility and function was independent of microbiota, we next investigated the local inflammatory milieu 2h after renal I/R-injury. At this time point, granulocyte influx did not occur yet (Figure 2A) and renal damage was similar between the groups (Figure 2B-C). Interestingly, renal inflammatory response was significantly lower as reflected by decreased mRNA levels of TNF-α, IL-6, MCP-1 and MIP-2α in antibiotic-treated mice (Figure 2D-G). Only levels of KC were similar between control and microbiota-depleted mice (Figure 2H). Upon injury, the sources of cytokines/chemokines release are TECs and renal resident leukocytes, among which macrophages constitute the major population[8, 25]. After 2h of reperfusion the level of macrophages were quantified by expression of classical renal macrophage marker F4/80 on mRNA and at protein level (Figure 2I, J). Strikingly, F4/80 expression was profoundly lower in commensal-depleted mice.

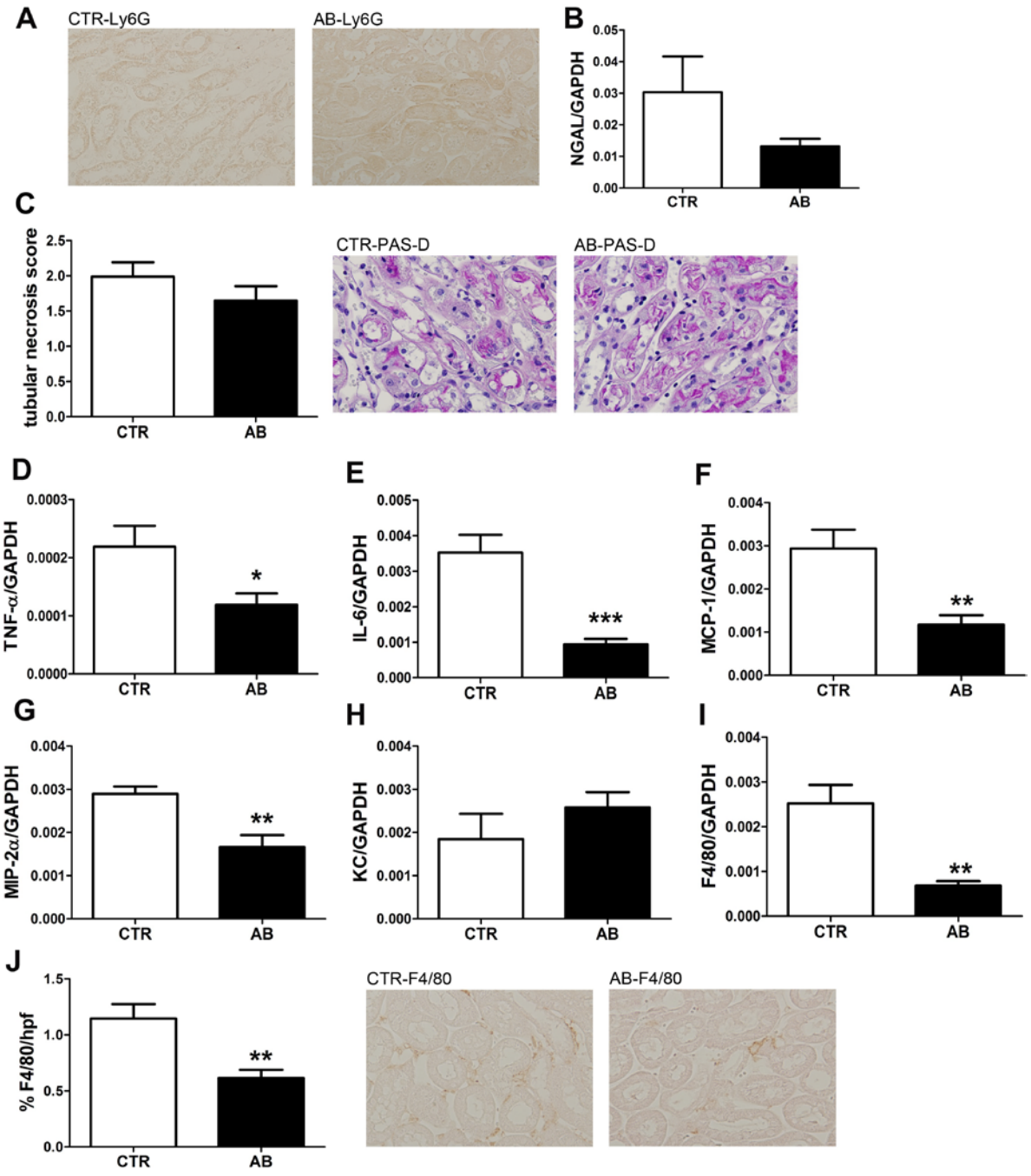

Figure 2: **Decreased levels of renal inflammatory mediators early after ischemic damage in commensal-depleted mice.** The number of granulocytes was determined by Ly6G$^+$ staining (magnification 400x) of kidney tissue (A). Renal damage was assessed by quantification of NGAL mRNA level in total kidney homogenates (B) and by semi-quantitative scoring the percentage of necrotic tubules in PAS-D-stained sections (magnification 400x) (C). Inflammatory mediators; TNF-α (D), IL-6 (E), MCP-1 (F), MIP-2α (G) and KC (H) were measured on mRNA level in kidney homogenates. F4/80 expression was determined by mRNA level in kidney homogenates (I) and by F4/80 staining (magnification 200x) of kidney tissue which was digitally analysed and presented as percentage of staining intensity per high-power field (J). Data are presented as mean ± SEM. The two-tailed unpaired *t* test was used in all graphs (n=8/9 per group). **P*<0.05, ***P*<0.01 and *** *P*<0.005.

F4/80+ renal resident macrophages from commensal-depleted mice are less mature and produce less MIP-2α in the early hours of I/R-injury

FACS analysis on renal resident macrophages at steady state and after 2h of reperfusion revealed that F4/80 expression was selectively reduced on F4/80+ population in commensal-depleted mice (Figure 3A-C), while no changes occurred for CD11B and CD11C, myeloid and dendritic cell markers, respectively (Figure S2A-B). Next, we sorted F4/80+ macrophages at 2h of reperfusion and determined their chemokine expression. Strikingly, we found that chemokines MCP-1 and MIP-2α were both decreased in F4/80+ macrophages isolated from antibiotic-treated mice (Figure 3D-F). KC and MIP-2α are the main chemokines that are produced within the early hours of reperfusion and known to attract granulocytes from circulation[7]. However, we could not detect any KC in these macrophages, which indicates that KC is not produced by these cells at 2h of reperfusion. In conclusion, the reduced granulocyte influx after 24h of reperfusion in the antibiotic-treated mice can be explained by the less mature F4/80+ macrophages that produce reduced levels of MIP-2α at 2h of reperfusion.

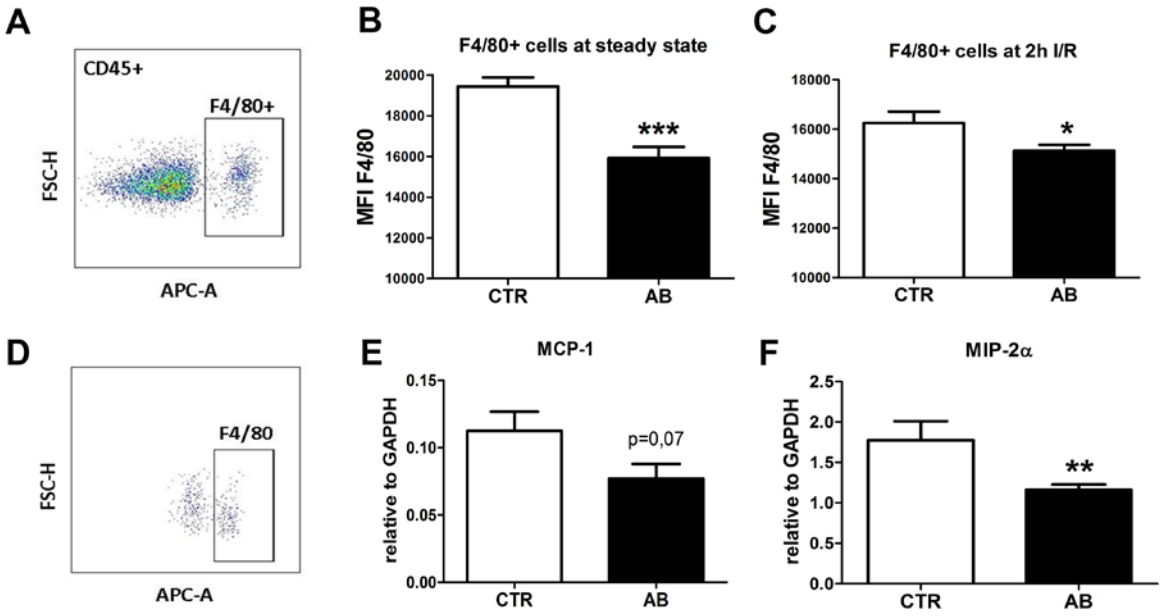

Figure 3: **F4/80+ renal resident macrophages from commensal-depleted mice are less mature and produce less MIP-2α in the early hours of I/R-injury.** A representative FACS plot showing the F4/80+ population on the CD45+ cells (A). MFI of F4/80 on F4/80+ cells at steady state (B) and after 2h of reperfusion. FACS plot illustrating the F4/80+ macrophages after 2h of I/R-injury that is sorted (D). MCP-1 (E) and MIP-2α (F) mRNA level in F4/80+ resident macrophages. Data are presented as mean ± SEM. The two-tailed unpaired *t* test was used in all graphs (n=5 per group). **P*<0.05, ***P*<0.01 and *** *P*<0.005.

Expression levels of F4/80 and CX3CR1 in renal resident macrophages is reduced by commensal depletion and restored after reintroduction of microbiota

We analysed renal resident macrophages under steady state and found that next to the F4/80 expression (Figure 4A), the chemokine receptors CX3CR1 and CCR2 (Figure 4C-F), which are important in trafficking of macrophages, were both reduced after depletion of gut microbiota[27, 28]. Despite the reduced expression of the chemokine receptors, the percentage of F4/80+ cells in the total population of CD45+ cells was unchanged in antibiotic-treated mice (Figure 4B). In order to investigate whether microbiota was responsible for affecting expression levels of these markers, we reintroduced microbiota by transplanting healthy fecal samples in mice initially depleted from commensals (Ftx-group). Fecal transplantation restored to a certain extent the expression rate of F4/80 and CX3CR1 but not of CCR2 (Figure 4A, C-F). The reduced levels of F4/80, CX3CR1 and CCR2 were only found on the F4/80+ cells and not on CD11B+ cells (Figure S3A, D, F), because they did not occur in the CD11B+ F4/80- population (Figure S3E, G). In the total population of CD11B, the majority of cells are F4/80 negative (~60%) while the F4/80+ proportion is around 40% (Figure S3B-C). Therefore, changes in the F4/80+ population are no longer visible in the total CD11B+ population. Together these findings show that in the absence of microbiota, only the F4/80+ population of renal resident macrophages are less mature.

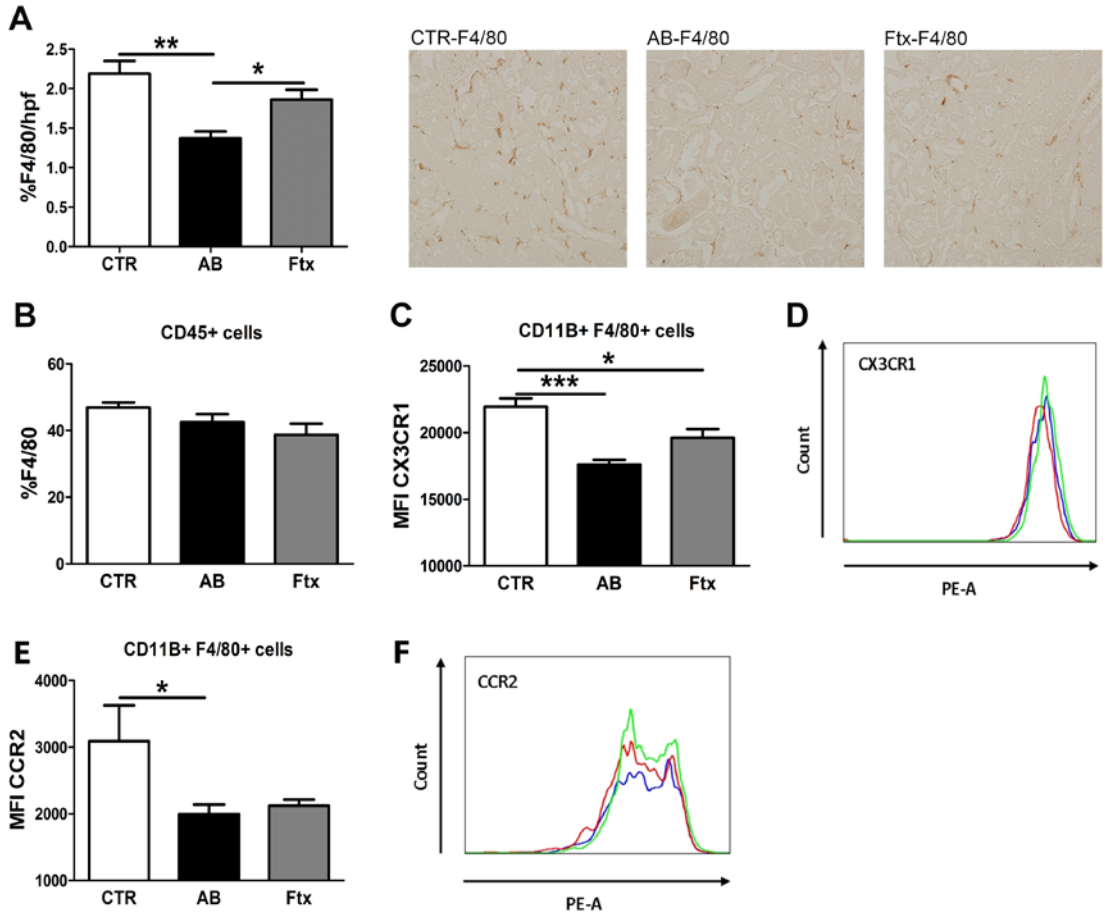

Figure 4: **Expression levels of F4/80 and CX3CR1 in renal resident macrophages is reduced by gut flora depletion and restored after reintroduction of microbiota.** F4/80 positivity was determined by F4/80 staining (magnification 200x) of kidney tissue which was digitally analysed and presented as percentage of staining intensity per high-power field of kidney tissue (A). The percentage of F4/80 positive cells in the total gate of CD45 positive cells was measured by Flow Cytometry (B). MFI of CX3CR1 positive cells (C) and its FACS plot (D) in CD11B+ F4/80+ gate from control (green), antibiotic (red) and Ftx group (blue). MFI of CCR2 (E) and its FACS plot (F) on CD11B+ F4/80+ macrophages from control (green), antibiotic (red) and Ftx group (blue). Data are presented as mean ± SEM. One-way ANOVA followed by Bonferroni's multiple comparison test was used in all graphs (n=5/6 per group). **P*<0.05, ***P*<0.01 and *** *P*<0.005.

Migration capacity and expression levels of F4/80, CX3CR1 and CCR2 are reduced in BM-monocytes from commensal-depleted mice

Maintenance of renal resident macrophages under steady conditions depends on a constant supply of mature monocytes from the bone marrow[29, 30] and relies on the expression of the chemokine receptor CX3CR1. Whereas the chemokine receptor CCR2 is important for recruitment of monocytes during inflammatory conditions[31]. Therefore, we questioned

whether BM-monocytes from antibiotic-treated mice, similarly to F4/80+ renal resident macrophages, would display lower levels of these receptors and, hence, poorer migratory capacity. As illustrated in figure 5A-D, F4/80, CX3CR1 and CCR2 MFI levels were markedly reduced in BM-monocytes from commensal-depleted mice compared to control mice. In line with these findings, depletion of microbiota impaired the migration ability of BM-monocytes towards CCL2 and CX3CL1 ligands (Figure 5E-F). Fecal transplantation reversed the phenotype, as shown by higher expression levels of F4/80, CX3CR1 and CCR2 on BM-monocytes in the Ftx-group compared to antibiotic-treated mice (Figure 5A-D). Overall, these findings show that F4/80+ BM-monocytes, similar to F4/80+ renal resident macrophages require signals from the gut to terminally differentiate.

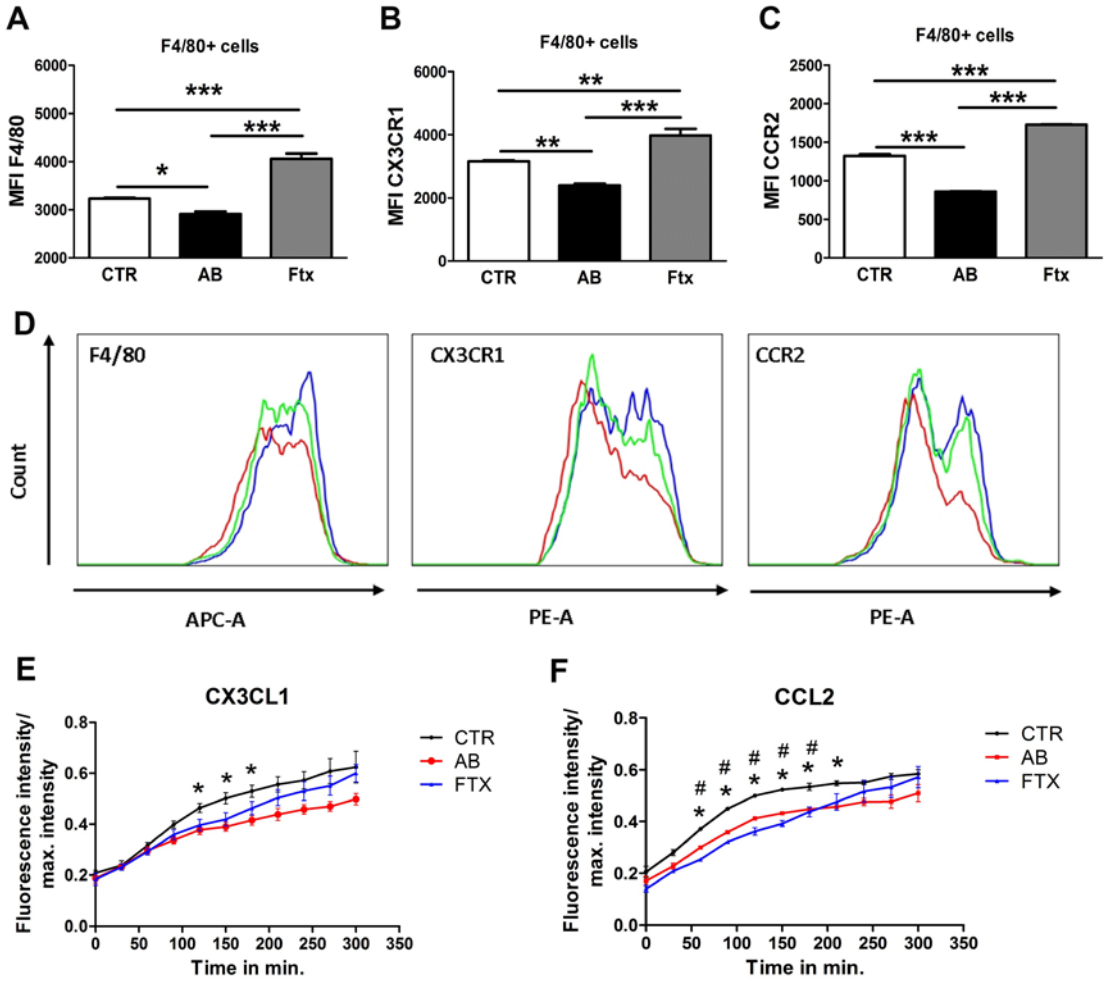

Figure 5: **Migration capacity and expression levels of F4/80, CX3CR1 and CCR2 are reduced in BM-monocytes from commensal-depleted mice.** MFI of F4/80 (A), CX3CR1 (B) and CCR2 (C) was determined on isolated F4/80+ BM-monocytes and its FACS plots (D) from control (green), antibiotic (red) and Ftx group (blue). Migration capacity of BM-monocytes with CX3CL1 (E) and CCL2 (F) as a chemoattractant was

quantified in transwell migration system (D). Data are presented as mean ± SEM. One-way ANOVA followed by Bonferroni's multiple comparison test was used in all graphs (n=4 per group). Significant difference between control and antibiotic group is indicated with a '*' and differences between control and the Ftx-group are given with a '#'. *P<0.05, **P<0.01 and *** P<0.005.

Reintroduction of microbiota into antibiotic-treated mice abolishes the protective effect of microbiota depletion upon renal I/R-injury

Since the low maturation status of F4/80+ renal macrophages that was observed in antibiotic-treated mice was reversed by fecal transplantation (Figure 4A), we wanted to investigate if reintroduction of commensals would also reverse response to renal I/R-injury. Indeed, we found no differences in tubular necrosis and NGAL levels between Ftx and control group while there was a great difference between control and antibiotic group (Figure 6A-B). Similarly, fecal transplantation reversed granulocyte influx to a certain extent (Figure 6C). To conclude, depletion of microbiota was indeed responsible for the protective effect against renal I/R-injury observed in antibiotic-treated mice (Figure 1).

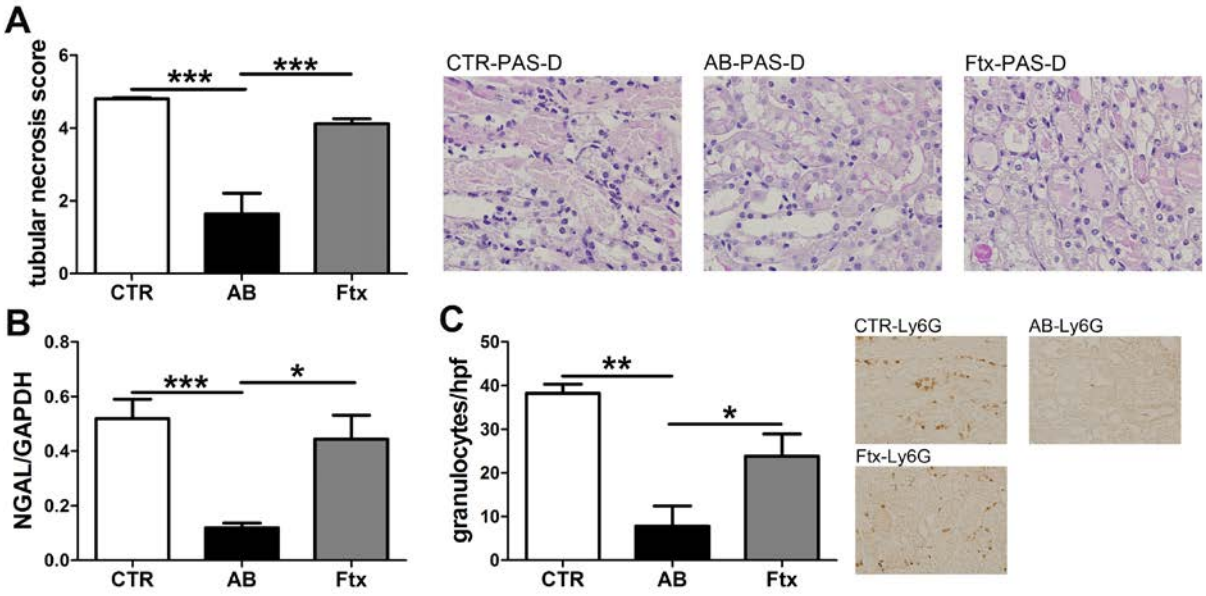

Figure 6: **Reintroduction of microbiota into antibiotic-treated mice abolishes the protective effect of microbiota depletion upon renal I/R-injury.** (A) Renal damage was assessed by semi-quantitative scoring of the percentage of necrotic tubules in PAS-D-stained sections (magnification 400x) and by determining NGAL mRNA level in total kidney homogenates (B). Influx of granulocytes was determined by scoring $Ly6G^+$ cells (magnification 400x) in the corticomedullary region of the kidney (C). Data are expressed as mean ± SEM. One-

way ANOVA followed by Bonferroni's multiple comparison test was used in all graphs (n=6/7/10 per group). *P<0.05, **P<0.01 and *** P<0.005.

Liver and splenic resident macrophages are less mature in commensal-depleted mice under steady condition

As we found a markedly decrease of F4/80 expression on renal resident macrophages from commensal-depleted mice under steady condition (Figure 4A), we questioned if resident macrophages of other organs are also affected by depletion of gut microbiota. We found reduced levels of F4/80 expression in liver and spleen tissue as well (Figure 7A-B). These immature resident macrophages in the liver provide an explanation for the preserved remote liver injury in commensal-depleted mice upon renal I/R-injury (Figure 1F-G). Apparently, gut microbiota do not only induce maturation of renal resident macrophages but also prime resident macrophages in liver and spleen.

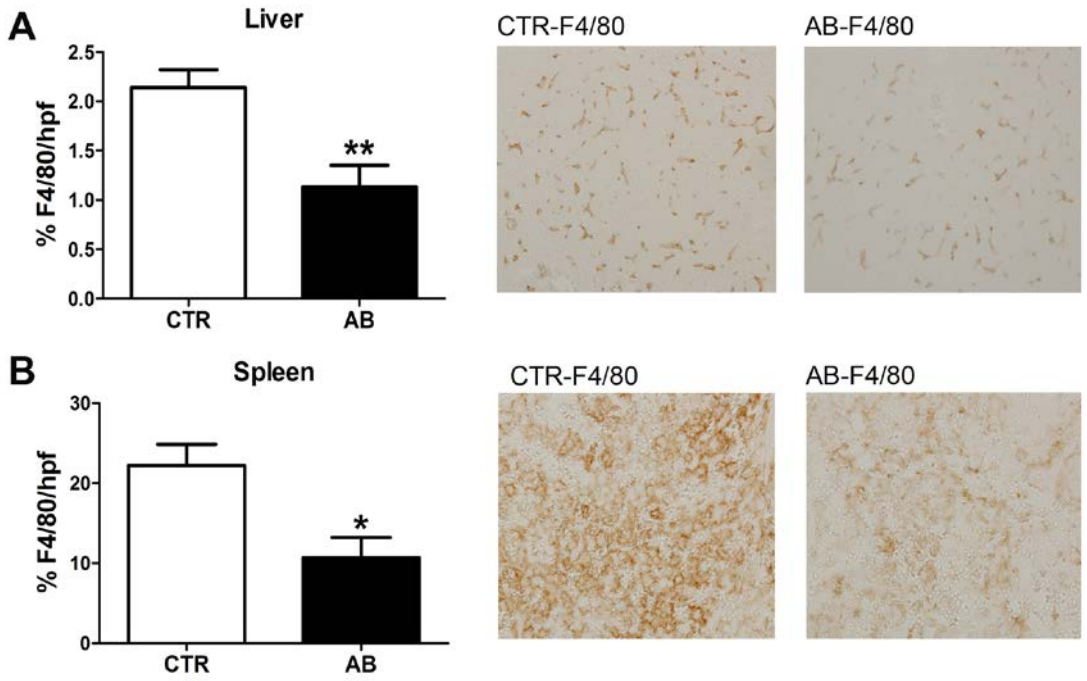

Figure 7: **Liver and splenic resident macrophages are less mature in commensal-depleted mice under steady condition.** F4/80 expression was determined by F4/80 staining (magnification 200x) of liver (A) and spleen (B) tissue and presented as percentage of staining intensity per high-power field. Data are expressed as mean ± SEM. Two-tailed unpaired *t* test was used in all graphs (n=4/6 per group). *P<0.05, **P<0.01.

Discussion

In the past decade it has become clear that microbiota play a fundamental role in shaping the immune system outside the gut. Increasing amount of studies show that alterations in gut microbiome affect the immune response to different inflammatory disorders[9, 10, 16]. However, little is known if similar processes occur during AKI. Here, we show mechanistic insight in the gut-kidney axis during AKI. Microbiota depletion was protective against renal I/R-injury as reflected by reduced renal dysfunction, damage and inflammation. Mechanistically, microbiota depletion affects macrophage/monocyte maturation status, release of chemokines (MCP-1 and MIP-2α) and primary functions like migration capacity. The reduced macrophage activity can explain the weaker early inflammatory response following I/R-injury. Protective effect of microbiota depletion against I/R-injury and macrophage alterations was reversed after reintroduction of microbiota following fecal transplantation. This study clearly shows that the microbiome prime macrophage functions that contribute to the initiation of renal inflammation and subsequent nephropathy following renal I/R-injury.

Clarke et al. was the first to show that microbiota affect systemic innate immunity since granulocytes were less efficient in killing *S. aureus* and *S. pneumoniae ex vivo* following antibiotic treatment[12]. In renal I/R-injury, several immune cells are known to contribute to the severity of kidney injury including granulocytes through the release of mediators and enzymes that potentially damage the surrounding tissue[24]. In our hands, granulocyte function (migration, phagocytosis capacity and oxidative burst) was not affected by antibiotic treatment. This observation is in line with a previous study in which no differences were found in phagocytic ability, reactive oxygen species production as well as cell surface adhesion and transmigration markers in granulocytes between control and antibiotic-treated mice[13]. These data implicate that the reduced granulocyte content in our antibiotic-treated mice was not due to impaired granulocyte functions but due to alterations preceding granulocyte influx. Indeed, 2h after I/R-injury (when granulocyte influx is not yet observed), renal inflammation was reduced in microbiota-depleted mice.

Dendritic cells and macrophages are the major resident leukocytes in the kidney, which are present within the renal interstitium and are essential for homeostatic regulation of the kidney environment[32, 33]. These resident leukocytes are known to contribute to initiation of inflammation shortly after renal I/R[8] and depleting macrophages in the kidney prior to I/R

protects mice and rats against renal injury[34, 35]. Distinguishing dendritic cells and macrophages from each other is complicated since they have overlapping ontogeny, function and phenotype[28]. Therefore, we analysed renal expression of CD11B, CD11C, Ly6C (data not shown) and F4/80 in control and antibiotic-treated mice. We found a reduced expression of F4/80, CX3CR1 and CCR2 only in the F4/80+ resident macrophages, while no changes occurred in the expression of CD11B, CD11C and Ly6C markers. F4/80 is considered as the classical cell surface marker for mature resident macrophages[25, 36, 37] whereas CX3CR1 is involved in adhesion, migration and activation of leukocytes[27, 31, 38]. Although, tissue macrophages originate from the yolk sac[37], it is believed that the renewal of resident macrophages under steady conditions is dependent on a constant supply of CX3CR1+ monocytes from the bone marrow[29, 30]. Indeed, CX3CR1-deficient mice display 50% less renal resident macrophages[39]. BM-monocytes from commensal-depleted mice were also less mature as assessed by low expression of F4/80, CX3CR1 and CCR2 markers in the F4/80+ population and the migration capacity was also reduced. However, we did not find decreased numbers of F4/80+ resident macrophages in the kidneys. As we may speculate, there are several possibilities that can explain this observation; the life span of resident macrophages ranges from weeks to months, it might be that these resident macrophages are not renewed during the 2 week course of antibiotic treatment, so there is no need for migration of the precursors. This hypothesis would mean that the gut flora prime renal resident macrophages and BM-monocytes independently from each other, which is possible since circulation reaches both sites (kidneys and the bone marrow). On the other hand, trafficking receptors and the ability to migrate is indeed decreased in BM-monocytes from antibiotic-treated mice, however it is not completely diminished. Meaning that these precursors are still able to migrate (slowly) during renewal of resident macrophages. Longer treatment with antibiotics might result in decreased numbers of macrophages in the kidneys. The last possibility is the sum of both 2 scenario's. Gut microbiota may affect renal resident macrophages directly and indirectly via the bone marrow. In this way, 2 weeks of antibiotic treatment is still too short to see changes in macrophage numbers in the kidneys at steady state. The reduced maturation and activation state of macrophages due to microbiota depletion is supported by our finding that F4/80+ resident macrophages produce less MIP-2α after 2h of reperfusion. It is MIP-2α together with KC that are responsible for the optimal attraction of granulocytes into tissue. Thus, the reduced maturation status of resident macrophages observed in kidneys of antibiotic-treated mice explains the reduced MIP-2α production early after I/R, leading to

decreased granulocyte influx and eventually resulting in preserved renal function and attenuated renal damage.

It is of great interest to identify commensal-derived products which are responsible for priming systemic immunity in order to target it. Only one study has revealed that peptidoglycan can translocate from the gut to circulation. This statement was based on an experiment in which guts of germ-free mice were colonized with *E. coli* for 3 days[12]. Therefore, it is difficult to assume that this is also the case in normal conventional mice with complete functional microbiota. We also could not measure detectable levels of LPS and peptidoglycan in plasma of control mice (data not shown), suggesting that the levels are too low to be detected but sufficient for a tonic activation of monocytes/macrophages or that other gut-derived products are involved. Short chain fatty acids (SCFAs) are well known candidates that are end products of fermentation by intestinal bacteria. They are known to have anti-inflammatory properties[40]. Recently, it has been stated that SCFAs prevent renal I/R-injury[41]. The authors showed that only acetate treatment diminished cellular stress and inflammation in kidney I/R-injury. However, in this study SCFAs were used as a treatment in the presence of an intact microbiota, meaning that physiological concentrations of SCFAs in the circulation under steady conditions do not have substantial anti-inflammatory properties. Identifying microbiota-derived products that modulate systemic immunity remains imperative in future translational research. A first step towards identifying microbiota-derived products that are essential in priming monocytes/macrophages, would be reconstitution with specific groups of bacteria instead of total fecal transplantation.

Several studies have demonstrated a beneficial role for microbiota in priming immunity against eradicating infectious pathogens[9, 10, 14, 16]. In contrast, priming innate immunity is detrimental during pathological conditions in which inflammation is unwanted including lung[15] and kidney (our study) I/R-injury. We show that the microbiome prime macrophage functions to become fully mature and these mature macrophages contribute to the initiation of inflammation and subsequent nephropathy following renal I/R-injury. Of note, we are not advocating the use of antibiotics in the treatment of AKI but our findings do present first steps towards understanding how to tailor microbiota-related therapies.

Methods

Mice

C57Bl/6 (WT) mice were purchased from Janvier (Le Genest, France). Mice were housed under specific pathogen-free conditions receiving food and water *ad libitum*. 8-12 weeks old male mice were used in all experiments. The Animal care and Use Committee of the University of Amsterdam approved all animal experiments.

Microbiota depletion

Depletion of gut microbiota prior to renal I/R was achieved by administering mice broad-spectrum antibiotics via autoclaved (tap) drinking water (1 g/L ampicillin, 1 g/L metronidazole, 1 g/L neomycin (Sigma Aldrich) and 0.5 g/L vancomycin (Xellia Pharmaceuticals ApS)) supplemented with 1% (w/v) glucose. Control drinking water was prepared in the same way only without antibiotics. Drinking water was replaced twice a week. Two weeks of treatment with broad-spectrum antibiotic strategy did not affect gut integrity and led to a significant drop in microbial diversity as described before[16]. Antibiotic treatment did not lead to changes in body weight, temperature, baseline haematological indices and food intake (Figure S4B-G). Fluid intake was slightly less in antibiotic group, probably due to the unpleasant taste of antibiotic-containing drinking water but did not lead to dehydration and weight loss (Figure S4E). Baseline biochemical indices (ALAT, ASAT, creatinine, urea, LDH, albumin, total protein and triglycerides) were also comparable between control and antibiotic-treated mice (data not shown).

Fecal transplantation

A fresh fecal sample from a untreated mouse was collected and resuspended in 1 ml of NaCl. 100 µl of this suspension was administrated via oral gavage per mouse (Ftx-group). Fecal transplantation started one day after cessation of antibiotics and lasted for 3 days. Fecal transplantation protocol succeeded, as we did not detect any bacterial colonies in antibiotic-treated group while bacteria were abundantly present in control and Ftx fecal samples (Figure S4A).

Ischemia/reperfusion injury

I/R-injury was induced by unilateral or bilateral clamping (micro aneurysm clamps) of renal pedicles for 25 minutes under general inhalation anaesthesia (3% Isoflurane and oxygen). After removal of clamps, kidneys were inspected for restoration of blood flow. For analgesic purposes, mice received a subcutaneous injection of 0.1 mg/kg buprenorphine (Temgesic, Schering-Plough) 30 minutes prior surgery. Sham-operated animals underwent the same procedure except clamping of the renal pedicles. At the time of sacrifice, blood was collected by heart puncture in heparin-containing tubes followed by cervical dislocation. Kidneys were snap frozen in liquid nitrogen and stored at -80^{o}C or fixed in 10% formalin o/n prior to further processing.

Plasma biochemical analysis

Using standard autoanalyzer methods plasma levels of creatinine, urea, LDH, ASAT, and ALAT were determined by our hospital diagnostic facility.

Histology and immunohistochemistry

Formalin-fixed tissue was embedded in paraffin using standard procedures. Four-µm thick sections were cut and used for all stainings. For examining renal histology, sections were stained with periodic acid-Schiff reagents after diastase digestion (PAS-D). Injury to tubules was scored by a pathologist in a blinded fashion, semi-quantitatively by determining the percentage of affected tubules per 10 high power fields (hpf) according to the percentage of necrotic tubules in the corticomedullary region on a scale from 0 to 5 (0 = 0%, 1 = <10%, 2 = 10-25%, 3 = 25-50%, 4 = 50-75%, and 5 = >75%). For immunohistochemical staining, sections were boiled for 10 min in 10 mM sodium citrate buffer (pH 6.0) for F4/80, apoptosis, and proliferation detection or digested with a solution of 0.25% pepsine (Sigma Aldrich) in 0.01 M HCl for granulocyte detection. Subsequently, sections were incubated o/n with FITC-labelled anti-mouse Ly6G (BD Biosciences), rat anti-mouse F4/80 (Biorad) rabbit anti-mouse active caspase-3 (Cell Signaling Technology) or rabbit anti-human Ki67 (Neomarkers) to detect granulocytes, macrophages, apoptosis, or proliferation respectively. For CD11B and CD11C stainings, frozen renal section were stained with rat anti-mouse CD11B (BD Pharmingen) and hamster anti-mouse CD11C (eBioscience) o/n. Next, all sections were incubated for 30 min with relevant peroxidase-conjugated secondary antibodies and stained

using 3,3”-diaminobenzidine dihydrochloride (DAB). The number of Ly6G positive cells and the number of capase-3 and Ki67 positive TEC was counted in 10 non-overlapping high power fields. F4/80, CD11B and CD11C expression was digitally quantified with Image J software.

Reverse transcriptase-PCR

Total RNA was isolated from frozen renal tissue sections with Tri-reagent (Sigma Aldrich) according to the manufacturer’s protocol. RNA from sorted renal macrophages were isolated with a Qiagen RNA isolation kit according to manufacturer’s instruction (Qiagen). cDNA was synthesized using oligo-dt as primer. mRNA expression of NGAL, TNF-α, IL-6, MCP-1, MIP-2α, KC and F4/80 was measured by real-time quantitative reverse transcription-PCR (RT-PCR) performed on a Light Cycler 480 (Roche) with SYBR green PCR master mix (Bioline). Intensity of SYBR green dye was determined by linear regression analysis (LinRegPCR, developed by Heart Failure Research Center, Amsterdam, The Netherlands). Expression of specific genes were normalized to expression of the house keeping gene GAPDH. Primer sequences are provided in supplementary table 1.

Isolation of BM-granulocytes and monocytes

Femoral and tibial bone marrow was flushed with ice-cold sterile PBS with a 21-gauge needle above a 40 µm pore filter. Monocytes and granulocytes were isolated by means of magnetic labeling using Monocyte Isolation Kit and by anti-Ly6G Microbead Kit respectively, according to manufacturer’s instructions (Miltenyi Biotec).

Oxidative burst, migration and phagocytosis assay

The Amplex Red Hydrogen Peroxide kit (Invitrogen) was used to detect hydrogen peroxide release by BM-granulocytes and monocytes stimulated with PMA (100 ng/ml) (Sigma Aldrich) according to manufacturer’s instructions. Briefly, 20 µl of cell suspension ($0{,}75x10^6$ cells/ml) in Krebs Ringer phosphate buffer was added in a black 96-wells plate with clear bottom (Greiner bio-one) together with 100 µl reaction buffer containing 0,1 U/ml HRP and 50 µM Amplex Red reagent. The reaction was measured by 530 nm excitation and 585 nm emission with microplate reader CLARIOstar (BMG Labtech). For quantification of migration capacity of BM-granulocytes and monocytes, $5x10^6$ cells/ml were resuspended in HEPES buffer with 4 µg/ml Calceine-AM (Life Technologies) and incubated for 30 minutes

at 37ºC. Cells were washed once in PBS and resuspended ($1x10^6$ cells/ml) in HEPES buffer. Migration was measured by transwell system using 24-wells plate (BD Falcon) and Fluoroblok cell culture insert (BD Falcon) with pore size 3 µm (granulocytes) and 8 µm (monocytes). CX3CL1 and CCL2 in HEPES buffer were used as chemoattractants. Migration of cells was measured in time at 37ºC with excitation 485 nm and emission 530 nm with microplate reader CLARIOstar (BMG Labtech). Phagocytosis was measured with PHAGOTEST (Glycotope Biotechnology) according to the manufacturer's instructions. Briefly, 100 µl of heparinized whole blood was stained with opsonized FITC-labelled *E.coli* and incubated for 10 minutes in a 37 ºC water bath, while the negative control remained on ice. After phagocytosis was stopped, quenching solution was used to discriminate between surface bound and internalized *E.coli.* After lysing of the red blood cells and DNA staining, phagocytic capacity was measured by flow cytometry on a FACS Canto (BD Bioscience) and analyzed with FlowJo version 10.

FACS analysis and sorting of renal macrophages

After renal tissue mechanic and enzymatic digestion, leukocytes were isolated by density gradient centrifugation (330g 30 minutes, room temperature) using two density Percoll (GE Healthcare) medium of 40% (upper phase) and 80% (lower phase). Cells at the interface between 40% and 80% Percoll were harvested, washed, blocked with anti-Fc Receptor (Anti-Mouse CD16/CD32, eBioscience), and stained with antibodies anti-CD45.2-Percp Cy5.5, CD11b-FITC, F4/80-APC, CCR2-PE, CX3CR1-PE and Ly6C-PE (Biolegend). Stainings were visualized using a BD LSRFortessa™ cell analyser (BD Bioscience); DAPI (Sigma-Aldrich) was added to the cell suspension short before measurement to discriminate dead cells from alive cells. Gating strategies are illustrated in supplementary figure 5. Sorting of F4/80+ renal macrophages was performed on a SH800 Cell Sorter (Sony). In brief, kidneys (two kidneys per sample) were mechanically and enzymatically digested, subsequently leukocytes were isolated by Percoll gradient centrifugation and following Fc-Receptor blocking were stained with anti-CD45 labelled magnetic beads in order to isolate renal CD45+ cells, accordingly to manufacturer's instructions (Miltenyi Biotec). After isolation, CD45+ leukocytes were stained with anti-F4/80-APC, cells were gated for APC-positivity for sorting. Per kidney-pair between 10.000 and 20.000 macrophages were sorted in average. All flow cytometry data were analyzed using FlowJo v10 (Ashland, OR).

Whole blood composition

Absolute numbers of white blood cells and % of lymphocytes, monocytes and granulocytes in whole blood were determined with Scil Vet abc Plus+ (HORIBA Medical) in 30µl of heparinized blood sample.

Statistical analyses

All statistical analyses were performed using Graphpad Prism version 5 software. Comparisons between two groups were analysed using the two-tailed unpaired *t* test. One-way ANOVA followed by Bonferroni's multiple comparison test was used for comparison between more than 2 groups. Results are expressed as mean ± SEM. Values of $P<0.05$ were considered to represent a statistically significant difference.

Acknowledgments

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References

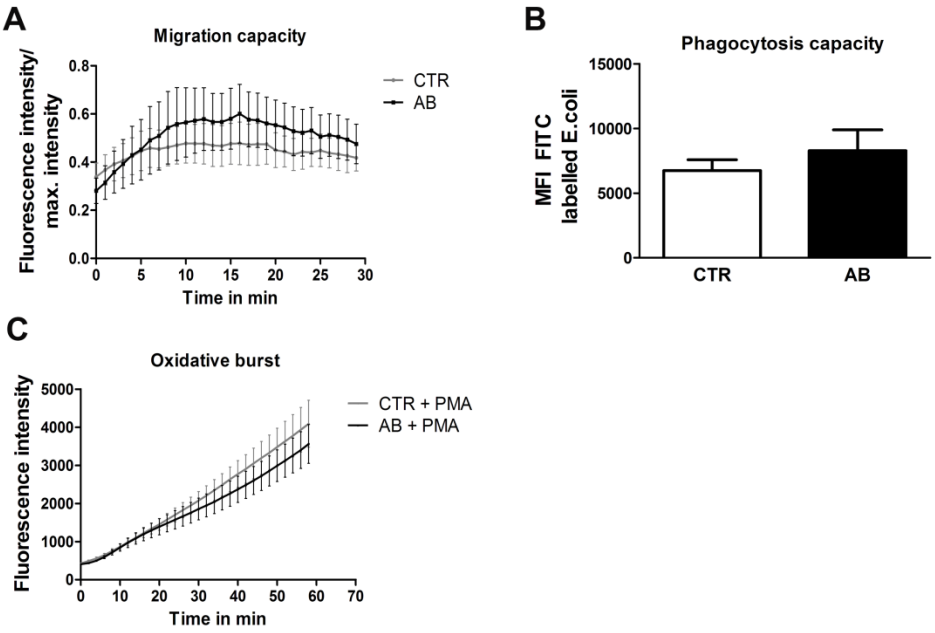
1. Gill, N, Nally, JV, Jr., Fatica, RA: Renal failure secondary to acute tubular necrosis: epidemiology, diagnosis, and management. *Chest,* 128**:** 2847-2863, 2005.
2. Le Dorze, M, Legrand, M, Payen, D, Ince, C: The role of the microcirculation in acute kidney injury. *Current opinion in critical care,* 15**:** 503-508, 2009.
3. Leemans, JC, Kors, L, Anders, HJ, Florquin, S: Pattern recognition receptors and the inflammasome in kidney disease. *Nature reviews Nephrology,* 10**:** 398-414, 2014.
4. Leemans, JC, Stokman, G, Claessen, N, Rouschop, KM, Teske, GJ, Kirschning, CJ, Akira, S, van der Poll, T, Weening, JJ, Florquin, S: Renal-associated TLR2 mediates ischemia/reperfusion injury in the kidney. *The Journal of clinical investigation,* 115**:** 2894-2903, 2005.
5. Bakker, PJ, Butter, LM, Claessen, N, Teske, GJ, Sutterwala, FS, Florquin, S, Leemans, JC: A tissue-specific role for Nlrp3 in tubular epithelial repair after renal ischemia/reperfusion. *The American journal of pathology,* 184**:** 2013-2022, 2014.
6. Pulskens, WP, Teske, GJ, Butter, LM, Roelofs, JJ, van der Poll, T, Florquin, S, Leemans, JC: Toll-like receptor-4 coordinates the innate immune response of the kidney to renal ischemia/reperfusion injury. *PloS one,* 3**:** e3596, 2008.
7. Stroo, I, Stokman, G, Teske, GJ, Raven, A, Butter, LM, Florquin, S, Leemans, JC: Chemokine expression in renal ischemia/reperfusion injury is most profound during the reparative phase. *International immunology,* 22**:** 433-442, 2010.
8. Akcay, A, Nguyen, Q, Edelstein, CL: Mediators of inflammation in acute kidney injury. *Mediators of inflammation,* 2009**:** 137072, 2009.
9. Abt, MC, Osborne, LC, Monticelli, LA, Doering, TA, Alenghat, T, Sonnenberg, GF, Paley, MA, Antenus, M, Williams, KL, Erikson, J, Wherry, EJ, Artis, D: Commensal bacteria calibrate the activation threshold of innate antiviral immunity. *Immunity,* 37**:** 158-170, 2012.
10. Benson, A, Pifer, R, Behrendt, CL, Hooper, LV, Yarovinsky, F: Gut commensal bacteria direct a protective immune response against Toxoplasma gondii. *Cell host & microbe,* 6**:** 187-196, 2009.
11. Clarke, TB: Early innate immunity to bacterial infection in the lung is regulated systemically by the commensal microbiota via nod-like receptor ligands. *Infection and immunity,* 82**:** 4596-4606, 2014.
12. Clarke, TB, Davis, KM, Lysenko, ES, Zhou, AY, Yu, Y, Weiser, JN: Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity. *Nature medicine,* 16**:** 228-231, 2010.
13. Deshmukh, HS, Liu, Y, Menkiti, OR, Mei, J, Dai, N, O'Leary, CE, Oliver, PM, Kolls, JK, Weiser, JN, Worthen, GS: The microbiota regulates neutrophil homeostasis and host resistance to Escherichia coli K1 sepsis in neonatal mice. *Nature medicine,* 20**:** 524-530, 2014.
14. Ichinohe, T, Pang, IK, Kumamoto, Y, Peaper, DR, Ho, JH, Murray, TS, Iwasaki, A: Microbiota regulates immune defense against respiratory tract influenza A virus

infection. *Proceedings of the National Academy of Sciences of the United States of America,* 108**:** 5354-5359, 2011.
15. Prakash, A, Sundar, SV, Zhu, YG, Tran, A, Lee, JW, Lowell, C, Hellman, J: Lung Ischemia-Reperfusion is a Sterile Inflammatory Process Influenced by Commensal Microbiota in Mice. *Shock,* 44**:** 272-279, 2015.
16. Schuijt, TJ, Lankelma, JM, Scicluna, BP, de Sousa, EMF, Roelofs, JJ, de Boer, JD, Hoogendijk, AJ, de Beer, R, de Vos, A, Belzer, C, de Vos, WM, van der Poll, T, Wiersinga, WJ: The gut microbiota plays a protective role in the host defence against pneumococcal pneumonia. *Gut*, 2015.
17. Ochoa-Reparaz, J, Mielcarz, DW, Ditrio, LE, Burroughs, AR, Begum-Haque, S, Dasgupta, S, Kasper, DL, Kasper, LH: Central nervous system demyelinating disease protection by the human commensal Bacteroides fragilis depends on polysaccharide A expression. *Journal of immunology,* 185**:** 4101-4108, 2010.
18. Wu, HJ, Ivanov, II, Darce, J, Hattori, K, Shima, T, Umesaki, Y, Littman, DR, Benoist, C, Mathis, D: Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. *Immunity,* 32**:** 815-827, 2010.
19. Niwa, T: Role of indoxyl sulfate in the progression of chronic kidney disease and cardiovascular disease: experimental and clinical effects of oral sorbent AST-120. *Therapeutic apheresis and dialysis : official peer-reviewed journal of the International Society for Apheresis, the Japanese Society for Apheresis, the Japanese Society for Dialysis Therapy,* 15**:** 120-124, 2011.
20. Ranganathan, N, Patel, BG, Ranganathan, P, Marczely, J, Dheer, R, Pechenyak, B, Dunn, SR, Verstraete, W, Decroos, K, Mehta, R, Friedman, EA: In vitro and in vivo assessment of intraintestinal bacteriotherapy in chronic kidney disease. *ASAIO journal (American Society for Artificial Internal Organs : 1992),* 52**:** 70-79, 2006.
21. Ueda, H, Shibahara, N, Takagi, S, Inoue, T, Katsuoka, Y: AST-120 treatment in pre-dialysis period affects the prognosis in patients on hemodialysis. *Renal failure,* 30**:** 856-860, 2008.
22. Stroo, I, Claessen, N, Teske, GJ, Butter, LM, Florquin, S, Leemans, JC: Deficiency for the chemokine monocyte chemoattractant protein-1 aggravates tubular damage after renal ischemia/reperfusion injury. *PloS one,* 10**:** e0123203, 2015.
23. Dessing, MC, Pulskens, WP, Teske, GJ, Butter, LM, van der Poll, T, Yang, H, Tracey, KJ, Nawroth, PP, Bierhaus, A, Florquin, S, Leemans, JC: RAGE does not contribute to renal injury and damage upon ischemia/reperfusion-induced injury. *Journal of innate immunity,* 4**:** 80-85, 2012.
24. Bolisetty, S, Agarwal, A: Neutrophils in acute kidney injury: not neutral any more. *Kidney international,* 75**:** 674-676, 2009.
25. Austyn, JM, Gordon, S: F4/80, a monoclonal antibody directed specifically against the mouse macrophage. *European journal of immunology,* 11**:** 805-815, 1981.
26. Dessing, MC, Tammaro, A, Pulskens, WP, Teske, GJ, Butter, LM, Claessen, N, van Eijk, M, van der Poll, T, Vogl, T, Roth, J, Florquin, S, Leemans, JC: The calcium-binding protein complex S100A8/A9 has a crucial role in controlling macrophage-mediated renal repair following ischemia/reperfusion. *Kidney international,* 87**:** 85-94, 2015.

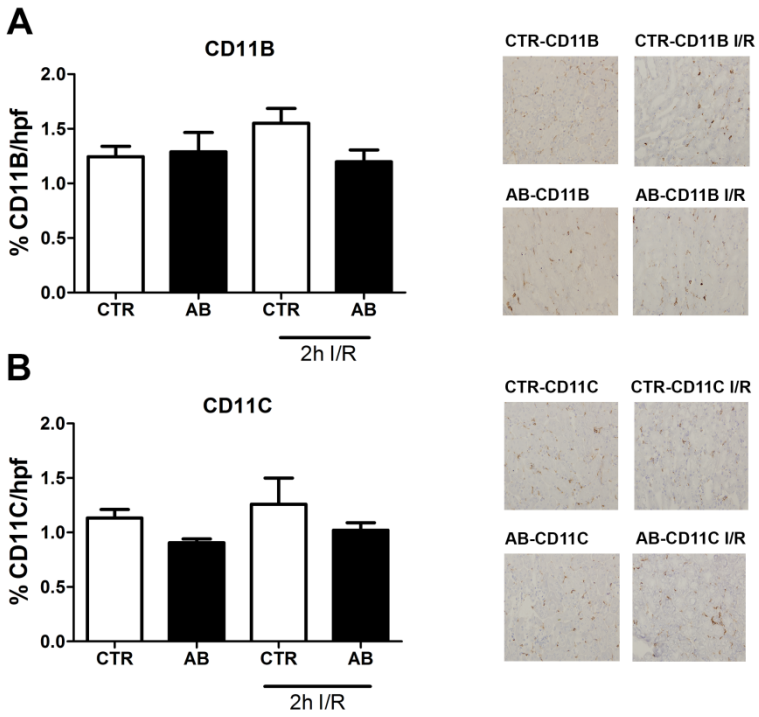
27. Umehara, H, Goda, S, Imai, T, Nagano, Y, Minami, Y, Tanaka, Y, Okazaki, T, Bloom, ET, Domae, N: Fractalkine, a CX3C-chemokine, functions predominantly as an adhesion molecule in monocytic cell line THP-1. *Immunology and cell biology,* 79**:** 298-302, 2001.
28. Kawakami, T, Lichtnekert, J, Thompson, LJ, Karna, P, Bouabe, H, Hohl, TM, Heinecke, JW, Ziegler, SF, Nelson, PJ, Duffield, JS: Resident renal mononuclear phagocytes comprise five discrete populations with distinct phenotypes and functions. *Journal of immunology,* 191**:** 3358-3372, 2013.
29. Gordon, S, Pluddemann, A, Martinez Estrada, F: Macrophage heterogeneity in tissues: phenotypic diversity and functions. *Immunological reviews,* 262**:** 36-55, 2014.
30. Gordon, S, Taylor, PR: Monocyte and macrophage heterogeneity. *Nature reviews Immunology,* 5**:** 953-964, 2005.
31. Geissmann, F, Jung, S, Littman, DR: Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity,* 19**:** 71-82, 2003.
32. Bonventre, JV, Yang, L: Cellular pathophysiology of ischemic acute kidney injury. *The Journal of clinical investigation,* 121**:** 4210-4221, 2011.
33. Nelson, PJ, Rees, AJ, Griffin, MD, Hughes, J, Kurts, C, Duffield, J: The renal mononuclear phagocytic system. *Journal of the American Society of Nephrology : JASN,* 23**:** 194-203, 2012.
34. Day, YJ, Huang, L, Ye, H, Linden, J, Okusa, MD: Renal ischemia-reperfusion injury and adenosine 2A receptor-mediated tissue protection: role of macrophages. *American journal of physiology Renal physiology,* 288**:** F722-731, 2005.
35. Jo, SK, Sung, SA, Cho, WY, Go, KJ, Kim, HK: Macrophages contribute to the initiation of ischaemic acute renal failure in rats. *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association,* 21**:** 1231-1239, 2006.
36. Schaller, E, Macfarlane, AJ, Rupec, RA, Gordon, S, McKnight, AJ, Pfeffer, K: Inactivation of the F4/80 glycoprotein in the mouse germ line. *Molecular and cellular biology,* 22**:** 8035-8043, 2002.
37. Schulz, C, Gomez Perdiguero, E, Chorro, L, Szabo-Rogers, H, Cagnard, N, Kierdorf, K, Prinz, M, Wu, B, Jacobsen, SE, Pollard, JW, Frampton, J, Liu, KJ, Geissmann, F: A lineage of myeloid cells independent of Myb and hematopoietic stem cells. *Science (New York, NY),* 336**:** 86-90, 2012.
38. Landsman, L, Bar-On, L, Zernecke, A, Kim, KW, Krauthgamer, R, Shagdarsuren, E, Lira, SA, Weissman, IL, Weber, C, Jung, S: CX3CR1 is required for monocyte homeostasis and atherogenesis by promoting cell survival. *Blood,* 113**:** 963-972, 2009.
39. Lionakis, MS, Swamydas, M, Fischer, BG, Plantinga, TS, Johnson, MD, Jaeger, M, Green, NM, Masedunskas, A, Weigert, R, Mikelis, C, Wan, W, Lee, CC, Lim, JK, Rivollier, A, Yang, JC, Laird, GM, Wheeler, RT, Alexander, BD, Perfect, JR, Gao, JL, Kullberg, BJ, Netea, MG, Murphy, PM: CX3CR1-dependent renal macrophage survival promotes Candida control and host survival. *The Journal of clinical investigation,* 123**:** 5035-5051, 2013.

40. Maslowski, KM, Vieira, AT, Ng, A, Kranich, J, Sierro, F, Yu, D, Schilter, HC, Rolph, MS, Mackay, F, Artis, D, Xavier, RJ, Teixeira, MM, Mackay, CR: Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. *Nature*, 461**:** 1282-1286, 2009.
41. Andrade-Oliveira, V, Amano, MT, Correa-Costa, M, Castoldi, A, Felizardo, RJ, de Almeida, DC, Bassi, EJ, Moraes-Vieira, PM, Hiyane, MI, Rodas, AC, Peron, JP, Aguiar, CF, Reis, MA, Ribeiro, WR, Valduga, CJ, Curi, R, Vinolo, MA, Ferreira, CM, Camara, NO: Gut Bacteria Products Prevent AKI Induced by Ischemia-Reperfusion. *Journal of the American Society of Nephrology : JASN*, 26**:** 1877-1888, 2015.

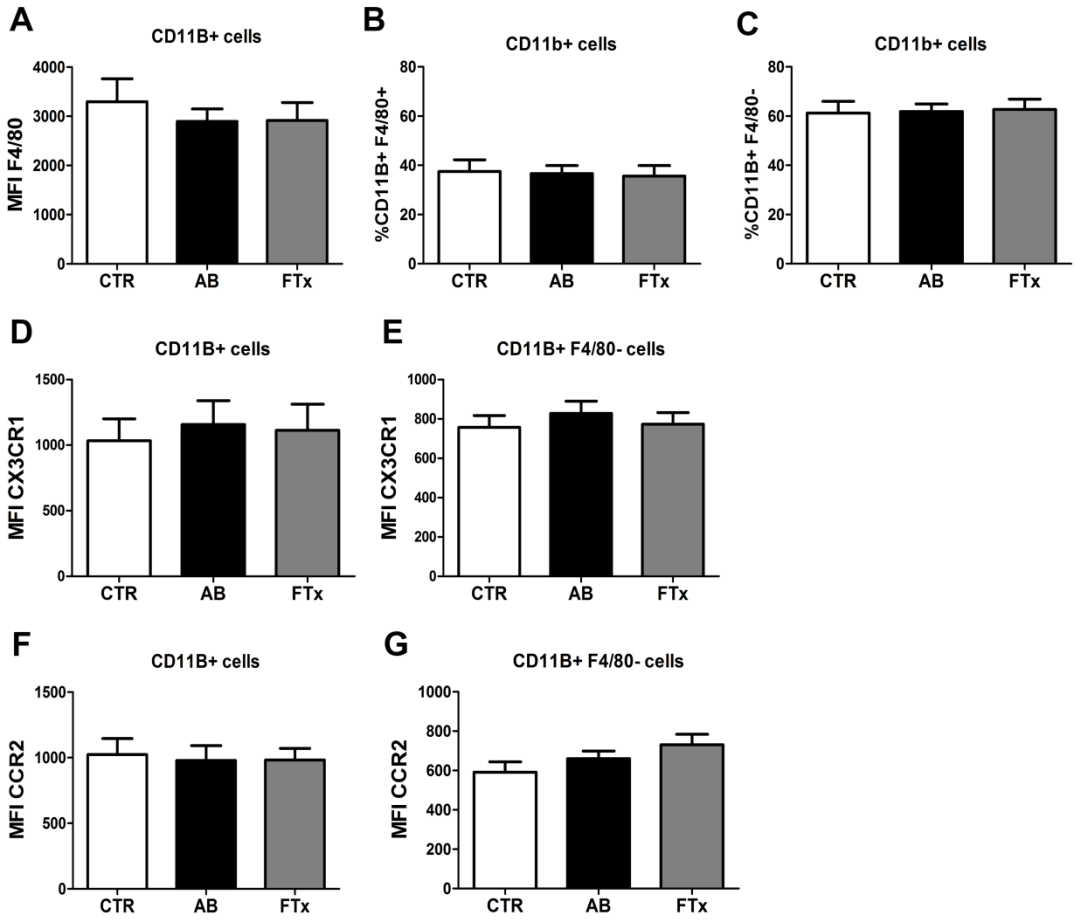
Supplementary information



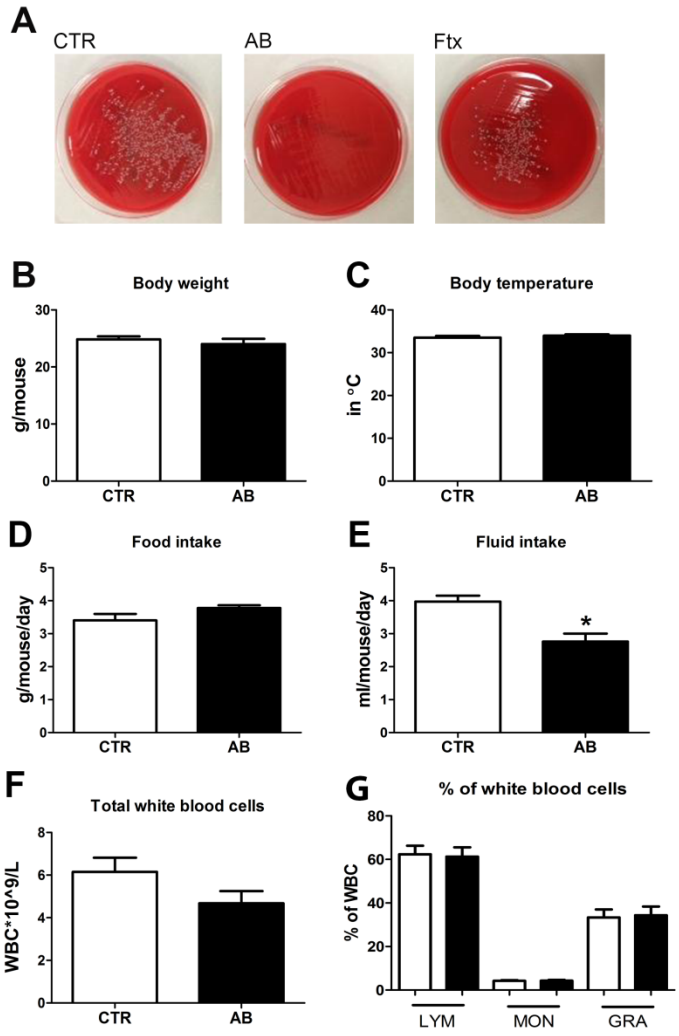
Supplementary figure 1: **Granulocyte function is not affected by depletion of gut microbiota.** BM-granulocytes were isolated and migration capacity (A), phagocytic ability (B) and oxidative burst (C) was quantified. Data are expressed as mean ± SEM (n=4 per group).



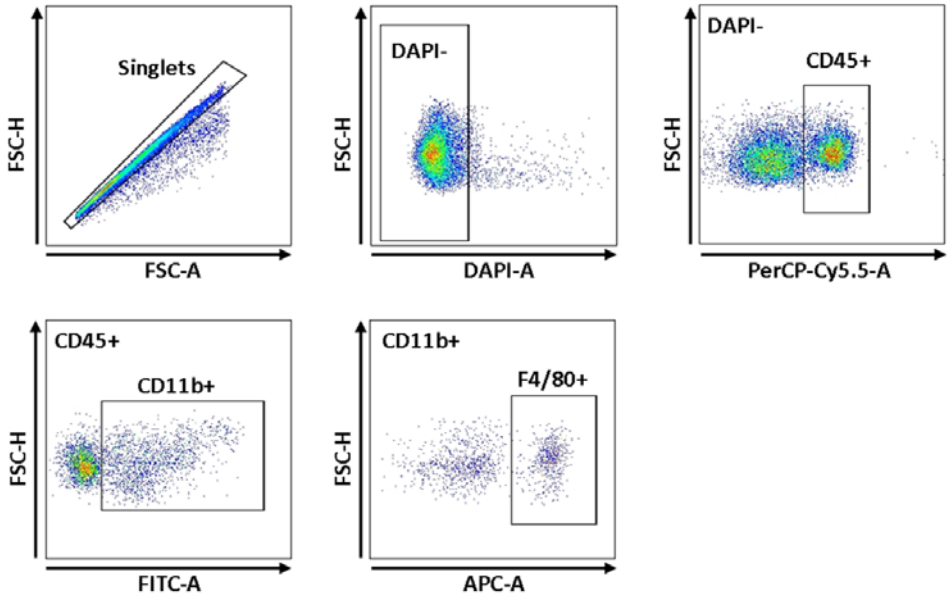
***Supplementary figure 2: Renal e*xpression level of CD11B and CD11C is not affected in commensal-depleted mice before and after 2h of I/R injury.** The percentage of CD11B (A) and CD11C (B) positivity was quantified per high-power field on kidney sections (magnification 200x). Data are expressed as mean ± SEM (n=5 per group).



Supplementary figure 3: **F4/80, CX3CR1 and CCR2 MFIs are not reduced in the total CD11B+ population after antibiotic treatment.** MFI of F4/80 (A), CX3CR1 (D) and CCR2 (F) on CD11B+ cells. MFI of CX3CR1 (E) and CCR2 (G) on CD11B+ F4/80- cells. Percentage of CD11B+ F4/80+ (B) and CD11B+ F4/80- (C) population in the CD11B gate. Data are expressed as mean ± SEM (n=8 per group).



Supplementary figure 4: **Antibiotic treatment did not lead to major changes in baseline parameters.** Presence/absence of fecal bacteria (A). Body weight (B), body temperature (C), food intake (D), fluid intake (E) and white blood cells (F-G) in control- and antibiotic-treated mice. Data are expressed as mean ± SEM. Two-tailed unpaired *t* test was used in graph E (n=8 per group). **P*<0.05.



Supplementary figure 5: **Gating strategy used during FACS analyses.** Cells were gated as follows: single cells, DAPI negative, CD45+, CD11B+ and F4/80+.

Tables

Supplementary table 1: Primers used for determination of specific genes

Gene	Forward primer	Reverse primer
NGAL	5'- GCCTCAAGGACGACAACATC	5'- CTGAACCATTGGGTCTCTGC
F4/80	5'- CTTTGGCTATGGGCTTCCAGTC	5'-GCAAGGAGGACAGAGTTTATCGTG
TNF-α	5'- TCGTAGCAAACCACCAAGTG	5'-CCTTGTCCCTTGAAGAGAACC
MCP-1	5'- CATCCACGTGTTGGCTCA	5'- GATCATCTTGCTGGTGAATGA
IL-6	5'- GCTACCAAACTGGATATAATCAGGA	5'- CCAGGTAGCTATGGTACTCCAGAA
MIP-2α	5'- CCCTGGTTCAGAAAATCATCC	5'- CTTCCGTTGACCCACAGC
KC	5'- ATAATGGGCTTTTACATTCTT	5'- AGTCCTTTGAACGTCTCTGTCC
GAPDH	5'- TGTCCGTCGTGGATCTGAC	5'- CCTGCTTCACCACCTTCTTG

Chapter 4

Release of extracellular DNA contributes to renal ischemia reperfusion injury through platelet activation and formation of neutrophil extracellular traps

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Abstract

Acute kidney injury is often the result of ischemia reperfusion injury, which leads to activation of coagulation and inflammation, resulting in necrosis of renal tubular epithelial cells. Platelets play a central role in coagulation and inflammatory processes, and it has been shown that platelet activation exacerbates acute kidney injury. However, the mechanism of platelet activation during ischemia reperfusion injury and how platelet activation leads to tissue injury are largely unknown. Here we found that renal ischemia reperfusion injury in mice leads to increased platelet activation in immediate proximity of necrotic cell casts. Furthermore, platelet inhibition by clopidogrel decreased cell necrosis and inflammation, indicating a link between platelet activation and renal tissue damage. Necrotic tubular epithelial cells were found to release extracellular DNA, which, in turn, activated platelets, leading to platelet-granulocyte interaction and formation of neutrophil extracellular traps ex vivo. Renal ischemia reperfusion injury resulted in increased DNA-platelet and DNA-platelet-granulocyte colocalization in tissue and elevated levels of circulating extracellular DNA and platelet factor 4 in mice. After renal ischemia reperfusion injury, neutrophil extracellular traps were formed within renal tissue, which decreased when mice were treated with the platelet inhibitor clopidogrel. Thus, during renal ischemia reperfusion injury, necrotic cell–derived DNA leads to platelet activation, platelet-granulocyte interaction, and subsequent neutrophil extracellular trap formation, leading to renal inflammation and further increase in tissue injury.

Introduction

Acute kidney injury (AKI) is a clinically important disorder associated with high mortality rates and increased risk of progression to end-stage renal disease. Despite major advances in knowledge of the underlying pathophysiology, a recent epidemiologic study shows that the incidence of AKI continues to grow.[1] AKI is often triggered by ischemia reperfusion (I/R), which leads to a complex interplay between coagulation, tubular epithelial and endothelial cell injury, and inflammation, resulting in renal tissue injury. Proximal tubular epithelial cells (TECs) in the corticomedullary region are especially susceptible to I/R due to their high oxygen demand.[2] After renal ischemia, a significant number of TECs will die either by necrosis or apoptosis.[3] Necrotic cells are highly immunostimulatory because they lead to the spilling of cell contents into the extracellular space, including danger-associated molecule patterns such as heat shock proteins, histones, high-mobility group box 1 proteins, and DNA.[4-6] Danger-associated molecule patterns alert the innate immune system to tissue damage. However, in the absence of an infectious etiology, the collateral damage caused by the initiation of this inflammatory response can be detrimental.

Apart from their pivotal role in hemostasis, platelets have been implicated in innate inflammatory processes as well. Accumulating evidence shows that platelets are major contributors to acute inflammation in the pathology of I/R injury.[7-9] It is now known that platelets express pattern recognition receptors through which they can get activated upon contact with danger-associated molecule patterns.[10-13] Platelet activation leads to platelet expression of P-selectin, which enables platelets to bind to other cells such as granulocytes, which can result in granulocyte activation and recruitment to sites of tissue damage.[14] It has been shown that granulocyte activation can lead to the formation of neutrophil extracellular traps (NETs).[15] Initially described as part of antimicrobial defense, recent studies indicate that NETs are also involved in the pathogenesis of noninfectious diseases.[16-19] Deoxyribonuclease 1 (DNase1) has a protective effect in vivo in murine models of myocardial I/R injury and acute lung injury as it dismantles the cytotoxic NET scaffold.[15,20] In addition, DNase1 could possibly dampen the immune responses to extracellular DNA derived from necrotic cells or NETs.

It has been shown that inhibition of platelet activation results in protection from renal I/R injury.[21] In the current study, we show that upon renal I/R, platelets are activated in close

proximity to necrotic casts and are probably mediated by the release of extracellular DNA by necrotic TECs (NTECs). In addition, for the first time, we show the presence of intrarenal cytotoxic NETs in the context of renal I/R, which can enhance platelet activation. We propose that after I/R, a vicious triad comprising NTECs, activated platelets, and NETs form a driving force in the pathophysiology of AKI.

Material and Methods

Animals

Pathogen-free 9- to 12-week-old male C57BL/6J wild-type (WT) mice were purchased from Charles River Laboratories. Age- and sex-matched WT mice were used in all experiments as a control. The Animal Care and Use Committee of the University of Amsterdam approved all experiments.

Murine renal ischemia reperfusion injury

Renal I/R injury was induced as described previously[22]. Renal I/R procedure was performed under general anesthesia (2% isoflurane). For analgesic purposes, mice received a subcutaneous injection of 0.1 mg/kg buprenorphin (Temgesic; Schering-Plough, Brussels, Belgium). Renal pedicles were clamped using non-traumatic vascular clips. After clamp removal, kidneys were inspected for restoration of blood flow. To maintain fluid balance, mice were supplemented with sterile 0.9% NaCl intraperitoneal. Mice were monitored every twelve hours the first day and daily one day post I/R.

(i) For the clamping time titration experiment, mice were subjected to bilateral clamping of renal pedicles for 0, 15, 20, or 25 minutes ($N = 15$, $N = 4$, $N = 12$, $N = 9$, respectively), followed by a 1-day reperfusion phase. (ii) To test the effect of DNase1 treatment on renal I/R injury, mice were subjected to bilateral clamping of renal pedicles for 25 minutes followed by a 1-day reperfusion phase. Mice were treated intraperitoneal with 200 µl DNase1 ($N = 8$) 20 mg/kg body weight (Roche Diagnostics, cat. no. 11284932001), or were treated with 200 µl vehicle (0.9% NaCl) ($N = 8$). (iii) To test the effect of clopidogrel on renal I/R injury, mice received clopidogrel (5 mg/kg per day) ($N = 6$) or vehicle (0.9% NaCl) ($N = 5$) by oral gavage

for 5 days. Renal I/R procedure was performed under general anesthesia (0.07 ml/10 g per mouse with a mixture containing 1.25 mg/ml midazolam (Actavis, Dublin, Ireland), 0.08 mg/ml fentanyl citrate, and 2.5 mg/ml fluanisone (VetaPharma Limited, Leeds, UK). Mice were killed after 24 hours.

To sacrifice, mice were anesthetized with 2% isoflurane followed by blood collection by heart puncture in heparine-coated tubes, and cervical dislocation. Kidneys were either snap-frozen in liquid nitrogen or formalin-fixed followed by paraffin embedment.

Plasma biochemical analysis

Murine renal function was determined by measuring creatinine and urea in plasma by enzyme reactions involving urease and creatinase and using standard autoanalyzer methods by our hospital research services.

Ex vivo platelet stimulations

Immortalized human tubular epithelial cells (HK2 cells) (0.4×10^6/ml) were washed 3 times with PBS and then taken up in PBS before they were repeatedly freeze-thawed in liquid nitrogen to obtain NTEC material. Platelet-rich plasma was obtained by centrifugation (180 rcf [relative centrifugal force] for 15 minutes) of human blood, and isolated platelets were obtained by adding acid citrate dextrose to platelet-rich plasma before centrifugation at 230 rcf for 15 minutes. Isolated platelets were then homogenized in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer and used for stimulation assays. Human platelets in whole blood were stimulated with necrotic cell material in the presence or absence of DNase1 (8 U/ml; Roche Diagnostics, Indianapolis, IN). For platelet stimulation with NETs, human granulocytes were isolated from healthy individuals, using Polymorphprep (Axis- Shield, Oslo, Norway) following the manufacturer's protocol. NETs were induced by stimulating granulocytes (2.5×10^5) with Phorbol 12-myristate 13-acetate (PMA) (100 nM) for 4 hours.[23] Granulocytes or NETs were then washed 3 times with PBS. Washed granulocytes or NETs were then incubated with human platelets (2.2×10^7) for 1 hour. Supernatant was then carefully transferred to an Eppendorf tube and spinned down in the presence of acid citrate dextrose ($Na_3C_3H_5O(COO)_3$ 85 mM, glucose 110 mM, $C_6H_8O_7$ 65 mM). Platelet-free supernatant was then used for mouse PF4 enzyme-linked immunosorbent assay.

Isolation of necrotic tubular epithelial cell-derived DNA

The obtained NTEC material was processed using QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions in order to obtain purified DNA and eluted in the appropriate elution buffer. DNA concentrations were determined with PICOgreen reagent (Quant-iTTM PICO greenH dsDNA Detection Kit; Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Fluorescence of dye bound to DNA was immediately determined by a fluorescence microplate reader (CLARIOstar, BMG LAB TECH, Ortenberg, Germany).

In vitro neutrophil extracellular trap experiments

Human granulocytes were isolated from normal individuals using Polymorphprep (AXIS-SHIELD PoC AS, Oslo, Norway) following the manufacturer's protocol. At a concentration of 100 nM, PMA served as a positive control for NET formation.[23] Granulocytes (4×10^5) in 24-well plates were stimulated with PMA, Roswell Park Memorial Institute culture medium, NTEC supernatant, platelets (3×10^7), platelets in combination with NTEC supernatant (volume ratio 1:3), platelets in combination with NTEC DNA (6 ng) or DNA buffer, or DNA buffer only, and then fixed on coverslips, permeabilized, and blocked for immunofluorescent staining. Cells were incubated with antibodies against neutrophil elastase (1:200 mouse anti-human; Dako, Glostrup, Denmark), CitH3 (dilution 1:200, rabbit anti-human citrulline 2+8+17; Abcam, Cambridge, UK), and DNA (1: 5000, Hoechst 33342, Thermo Fisher Scientific, Naarden, The Netherlands) and followed by species-specific secondary antibodies coupled with Alexa fluor dyes (Invitrogen). Cells were mounted in Vectashield Mounting media (Vector Laboratories, Burlingame, CA), and images were created with Leica DM5000B (Leica, Rijswijk, The Netherlands).

NET-positive cells were counted in 20 nonoverlapping fields with an original magnification ×20. Granulocytes (2.5×10^5) in 96-well plate were treated with PMA, unstimulated platelets (2.2×10^6 or 2.2×10^7), or platelets activated with thrombin receptor–activating peptide (15 μM; Sigma-Aldrich, St. Louis, MO) for 1 hour followed by washing and a 20-minute incubation with DNase1 (2 U/ml). Supernatant was then mixed 1:1 with PICO Green reagent (Quant-iTTM PICO greenH dsDNA Detection Kit, Invitrogen) following the manufacturers protocol. Fluorescence of dye bound to DNA was immediately determined

by a fluorescence microplate reader (CLARIOstar, BMG LAB TECH). For isolation of platelets, platelet-rich plasma (Sanquin Blood Supply, Amsterdam, The Netherlands) was centrifuged at 800*g* for 10 minutes at room temperature in the presence of acid citrate dextrose ($Na_3C_3H_5O(COO)_3$85 mM, glucose 110 mM, $C_6H_8O_7$ 65 mM); platelet pellets were resuspended in buffer B (NaCl 152 mM, $NaHCO_3$ 13.2 mM, glucose 7.16 mM, $MgCl_2$ $6H_2O$ 1.1 mM, KCL 2.9 mM, ethylenediamine tetraacetic acid 1 mM, pH 7.35) and counted by flow cytometry (FACS Calibur, Beckton Dickinson, Franklin Lakes, NJ).

(Immuno)histochemistry

Murine renal tissue was fixed and processed as previously described.[24] Paraffin-embedded sections were used for periodic acid–Schiff diastase staining, DNA Feulgen staining, and immunohistochemistry. The degree of tubular damage was assessed on periodic acid–Schiff diastase–stained 4-μm thick sections by scoring tubular cell necrosis in 10 nonoverlapping high-power fields (original magnification ×40) in the corticomedullary junction. The degree of injury was scored by a pathologist in a blinded fashion on a 5-point scale: 0 = no damage, 1 = 10% of the corticomedullary junction injured, 2 = 10% to 25%, 3 = 25% to 50%, 4 = 50% to 75%, and 5 = >75%. Tissue sections were incubated with specific antibodies for granulocytes (dilution, 1:1000 fluorescein isothiocyanate–labeled anti-mouse Ly6G (lymphocyte antigen 6G) monoclonal antibody (BD Biosciences–Pharmingen, Breda, The Netherlands), CitH3 (dilution 1:200, citrulline 2+8+17; Abcam), GPIba, clone SP219 (dilution 1:200, Spring Bioscience, Pleasanton, CA) and visualized with 3,3′-diaminobenzidine, Vector Red, or Vector Blue (Vector Labs). Ly-6G– and CitH3-positive cells were counted in 10 nonoverlapping high-power fields (original magnification ×40) in the corticomedullary junction. The percentage of positive GPIba staining in 10 nonoverlapping high-power fields was quantified using image analysis software (FIJI image J software). Both double-staining DNA Feulgen/platelet GPIba and triple-staining DNA Feulgen/platelet GPIba /Ly6G colocalization were analyzed and quantified with Leica imaging software.

Enzyme-linked immunosorbent assay

Cytokines were measured in platelet-free supernatant, plasma, and renal tissue homogenates. To obtain renal tissue homogenates, snap-frozen kidneys were diluted in lysis buffer (30 mM Tris, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 1% Triton X-100, and 1% protease inhibitor cocktail II [Sigma-Aldrich]), homogenized and incubated at 4 °C for 30 minutes. Homogenates were

subsequently centrifuged at 1500*g* at 4 °C for 15 minutes, and supernatants were stored at −80 °C until assays were performed. Mouse MPO (Hycult Biotech, Uden, The Netherlands) and PF4 (mouse PF4, cat no. DY595; R&D systems) were quantified with an enzyme-linked immunosorbent assay kit, following the manufacturer's instructions. Circulating mono- and oligonucleosomes were quantified with an enzyme-linked immunosorbent assay kit, following the manufacturer's instructions (Cell Death Detection ELISA PLUS, cat. no. 1774425, Roche Diagnostics). All tissue measurements were corrected for total protein concentration using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Total protein concentrations were measured by incubating 1µL of 10 times diluted homogenates for 30 minutes at 37°C in 500µL of bicinchoninic acid containing 4% of CuSO4, absorbance was measured at 570nm.

RNA purification and quantitative real-time polymerase chain reaction

Total RNA was extracted from snap-frozen renal tissue sections with Trizol reagent (Invitrogen and Life Technologies, Breda, The Netherlands) and converted to cDNA. mRNA level was analyzed by quantitative real-time polymerase chain reaction with SYBR green polymerase chain reaction master mix (Thermo Fisher Scientific). SYBR green dye intensity was analyzed with linear regression analysis. Specific gene expression was normalized to mouse housekeeping gene hypoxanthine-guanine phosphoribosyltransferase *(HPRT)*. The following murine primer sets were used: HPRT (for 5′-TCCTCCTCAGACCGCTTTT-3′, rev: 5′-CCTGGTTCATCATCGCTAATC-3′; neutrophil gelatinase–associated lipocalin (for 5′-GCCTCAAGGACGACAACATC-3′, rev: 5′-CTGAACCAATTGGGTCTCGC-3′); KIM-1 (for 5′-TGGTTGCCTTCCGTGTCTCT-, rev: 5′-TCAGCTCGGGAATGCACAA-3′).

Flow cytometry

Platelet activation and platelet leukocyte complex formation were determined in citrated blood by flow cytometry (FACS Calibur; Becton Dickinson, Franklin Lakes, NJ) using allophycocyanin-conjugated anti-CD61 APC (Dako), fluorescein isothiocyanate-conjugated anti-CD62p (Beckman, Coulter, Marseille, France), fluorescein isothiocyanate-labeled anti-PAC-1 (Becton Dickinson Biosciences) peridinin Chlorophyll Protein Complex-conjugated anti-CD61 (Becton Dickinson Biosciences, San Jose, CA, USA), and allophycocyanin-conjugated CD45 (Becton Dickinson Biosciences) in accordance with manufacturer's instructions.

Statistical analysis

All data sets were tested for their distribution prior to analyses. We performed statistical analysis by using an unpaired Student *t* test or analysis of variance for all *ex vivo* studies. *In vivo* differences between experimental groups were determined using the Mann-Whitney *U* test or Kruskal-Wallis test where appropriate. All analyses were done using GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA). Values are expressed as mean ± SEM. A P-value of < 0.05 was considered statistically significant.

Results

Renal ischemia/reperfusion leads to platelet activation in proximity of necrotic casts and inhibition of platelet activation ameliorates acute kidney injury.

Renal I/R injury was induced by clamping both renal arteries, leading to TEC necrosis, predominantly in the corticomedullary region. This also resulted in the formation of platelet aggregates in close proximity to necrotic casts (Figure 1a–c), indicating platelet activation in areas with tissue necrosis but not outside this specific region.

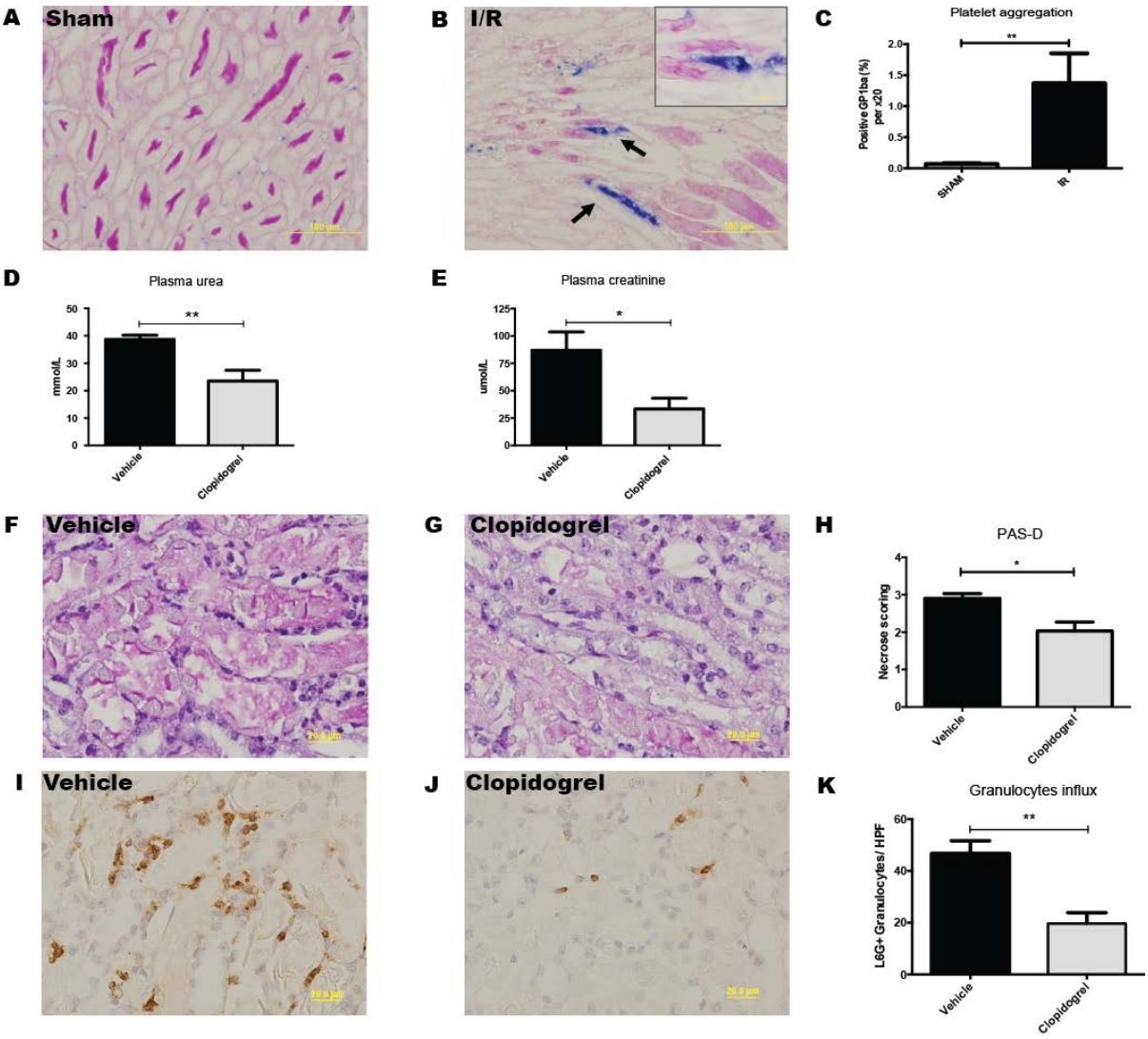


Figure 1 | Renal ischemia/reperfusion injury leads to platelet activation in proximity of necrotic casts, and inhibition of platelet activation ameliorates acute kidney injury renal. Representative photographs of periodic acid–Schiff diastase (PAS-D)/GPIba double-stained renal tissue sections of sham-operated mice (*N* = 6), original magnification ×20, bar = 100 μm (**a**) and mice subjected to renal ischemia/reperfusion (I/R) with 1-day reperfusion (*N* = 6), original magnification ×20, bar = 100 μm; inset, original magnification ×100, bar = 20 μm (**b**). Arrows in **b** indicate aggregated platelets (thus, activated platelets). (**c**) The percentage of positive GPIba staining in 10 nonoverlapping fields was quantified using FUJI image analysis software. Renal dysfunction of wild-type (WT) mice treated with vehicle (*N* = 5) compared with WT mice treated with clopidogrel (*N* = 6), as reflected by increased levels of urea (**d**) and creatinine (**e**). (**f**) Score for histopathology of renal tubular damage in WT mice and WT mice treated with clopidogrel after renal I/R injury using PAS-D–stained renal tissue sections; original magnification ×40, bars = 20 μm. (**g**,**h**) Representative photographs of PAS-D–stained renal tissue sections of the corticomedullary region. Neutrophil (lymphocyte antigen 6G [ly6-G] positive) influx in kidneys from control WT vehicle-treated (**i**) and WT clopidogrel-treated (**j**) mice 1 day after renal I/R or sham operation as assessed by immunohistochemistry and counted in at least 10 randomly selected high-power fields in the corticomedullary region (**k**). Original magnification ×40; bars = 20 μm. Data are mean ± SEM of mice per group. **P* < 0.05, ***P* < 0.001. HPF, high-power field.

Necrotic tubular epithelial cells stimulate platelet activation through release of extracellular DNA.

It has been shown that necrotic cells can induce an inflammatory reaction through activation of the innate immune system.[25] Moreover, platelets have been described to act as innate immune cells in inflammatory reactions.[26] They can interact with other immune cells, and it has been shown that platelets bind granulocytes and direct them into injured tissue.[14] Having shown that platelet aggregates colocalize with necrotic casts during I/R and that platelet activation con- tributes to postischemic cell necrosis and inflammation, we sought to study the effect of necrotic renal cells on platelet activation and platelet granulocyte interaction. In vitro, we necrotized TECs (NTECs) by repetitive freeze-thawing and then incubated human platelets with the supernatant. Flow cytometry analyses revealed a dose-dependent activation of platelets by NTECs, as illustrated by increased expression of P-selectin and binding to PAC-1 (Procaspase Activating Compound-1) (Figure 2a and b). Necrotic cells are known to release damage-associated molecular patterns that can alert the innate immune system through Toll-like receptor–mediated signaling.[27]

We hypothesized that the presence of extracellular DNA released from necrotic cells stimulates platelet activation. The presence of intact DNA in NTEC supernatant was confirmed by gel electrophoresis, and incubation with DNase1 resulted in the breakdown of DNA (Supplementary Figure S2). To determine the effect of NTEC-derived extracellular DNA on platelet activation, we stimulated platelets with NTEC supernatant treated with or without DNase1 or phosphate- buffered saline (PBS). Furthermore, we exposed platelets to purified DNA, isolated from NTEC supernatant. Platelets stimulated with DNase1-treated NTEC supernatant showed significant less activation (Figure 2c). In addition, isolated DNA from NTEC supernatant resulted in platelet activation (Figure 2d) indicating that extracellular DNA released from necrotic cells contributes to platelet activation.

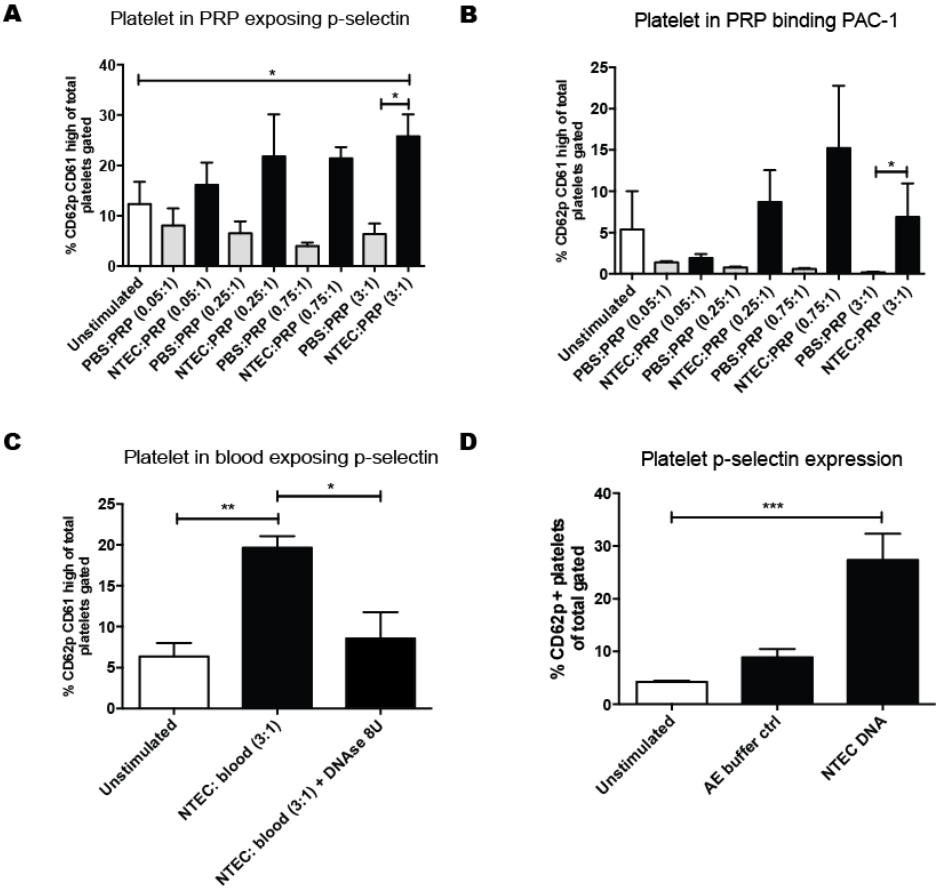


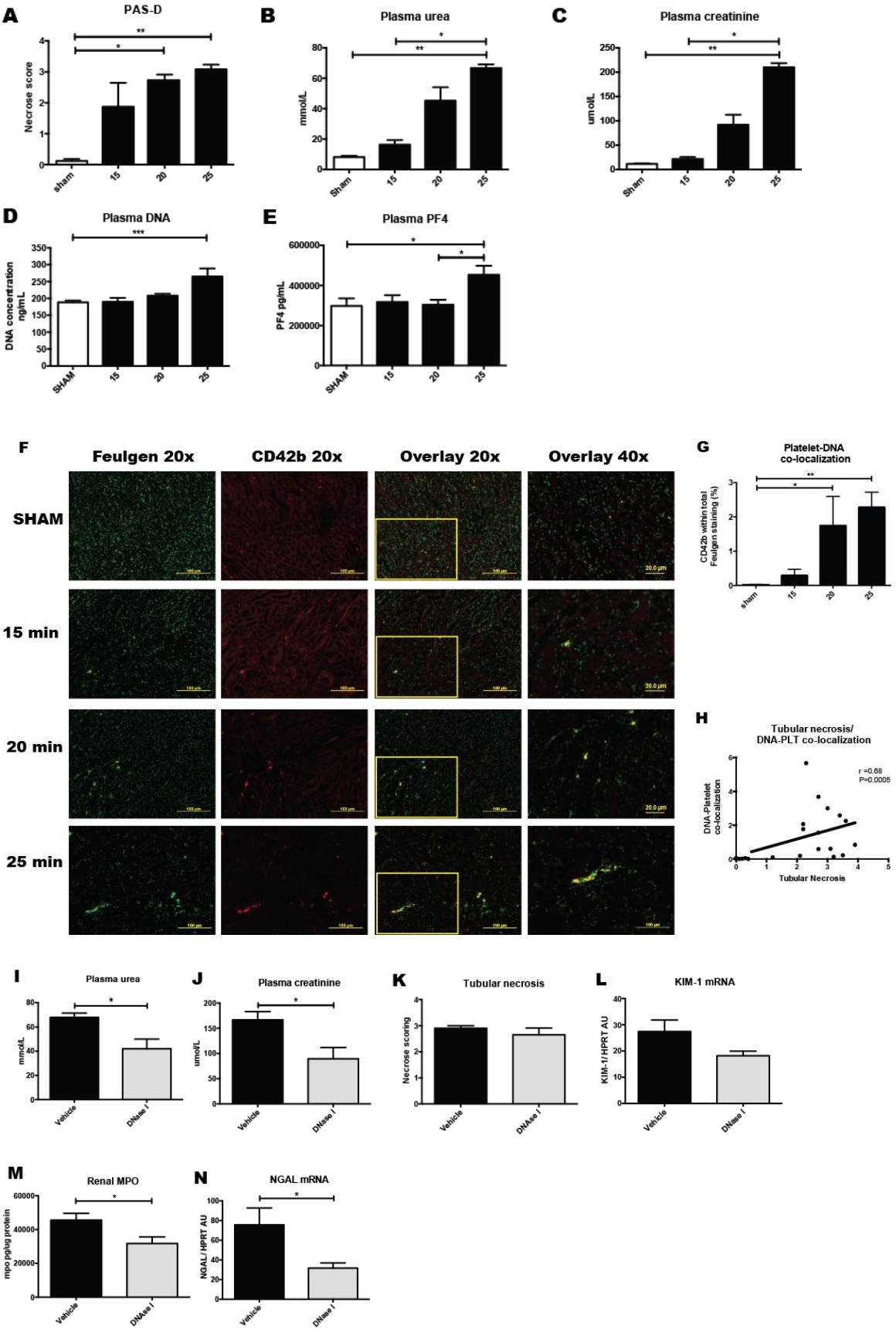
Figure 2 | Necrotic tubular epithelial cells stimulate platelet activation through release of extracellular DNA. *Ex vivo* dose-response stimulation of human platelets in platelet-rich plasma (PRP) with different volumes of supernatant derived from necrotic tubular epithelial cells (NTECs) (0.4×10^6 cells/ml), expressed as the ratio NTEC:PRP or different volumes of phosphate-buffered saline (PBS) control expressed as the ratio PBS control:PRP. Flow cytometry analysis for platelet CD62p exposure (**a**) or binding to platelet procaspase activating compound-1 (PAC-1) (**b**) (graph represents a minimum of 3 and a maximum of 8 independent experiments). (**c**) *Ex vivo* stimulation of human platelets in whole blood with necrotic supernatant and necrotic supernatant incubated with 8 U/ml DNase1. The ratio NTEC:blood is 3:1. (**d**) *Ex vivo* stimulation of washed human platelets stimulated with DNA isolated from NTEC supernatant. The relative amount of activated platelets is expressed as the % CD62p CD61 high of total platelets gated. Data are expressed as mean ± SEM. **P* < 0.05, ***P* < 0.001, ****P* < 0.0001.

In order to investigate the role of platelet activation in renal I/R, we treated mice with clopidogrel before subjecting them to renal I/R, leading to platelet inhibition, as evidenced by the prolonged bleeding time (Supplementary Figure S1). Treating mice with clopidogrel before bilateral renal artery clamping significantly reduced plasma urea and creatinine levels 24 hours after surgery compared with vehicle controls, demonstrating that platelet inhibition prevents the loss of renal function during AKI (Figure 1d and e). The preservation of renal function was associated with reduced tubular necrosis (Figure 1f–h) and decreased

granulocyte infiltrates at 24 hours after surgery compared with vehicle treatment (Figure 1i–k).

Renal ischemia/reperfusion leads to increased DNA release and platelet activation

Having established that extracellular DNA derived from necrotic cell material stimulates platelet activation, we hypothesized that renal I/R would lead to an increased release of extracellular DNA and subsequent platelet activation. To investigate this, we performed a clamping time titration. As expected, ischemia led to a clamping time–dependent increase of tubular cell necrosis (Figure 3a), plasma urea (Figure 3b), and creatinine (Figure 3c). In accordance, plasma DNA concentration (Figure 3d) and levels of platelet factor 4 (PF4), which is released upon platelet activation (Figure 3e), were significantly elevated at 25 minutes of ischemia. By staining renal tissue for DNA and platelets, we found significant platelet-DNA colocalization, which correlated with renal cell necrosis (Figure 3f–h). These results show that renal I/R injury leads to increased extracellular DNA leaking, leading to more platelet-DNA interaction and platelet activation in vivo in a time-dependent fashion. Next, we treated mice with DNase1 before and after renal I/R in an attempt to clear extracellular DNA leaking and to dampen subsequent platelet activation. DNase1 treatment did not sufficiently reduce plasma extracellular DNA and plasma PF4 levels, but it decreased plasma nucleosome levels (Supplementary Figure S3A and C). Although DNase1 treatment did not reduce tubular necrosis and renal expression of the kidney damage marker KIM-1 mRNA (Figure 3k and l), administration of DNase 1 did protect renal function, as reflected by creatinine and urea levels 1 day post-ischemia (Figure 3i and j). Protection of renal function did not correspond to a reduced granulocyte influx (not shown), but did coincide with a significant reduction of granulocyte activation markers in renal tissue, as shown by reduced levels of myeloperoxidase (MPO) protein levels and neutrophil gelatinase–associated lipocalin mRNA expression (Figure 3m and n).


A
PAS-D
Necrose score
sham
15
20
25
B
Plasma urea
mmol/L
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15
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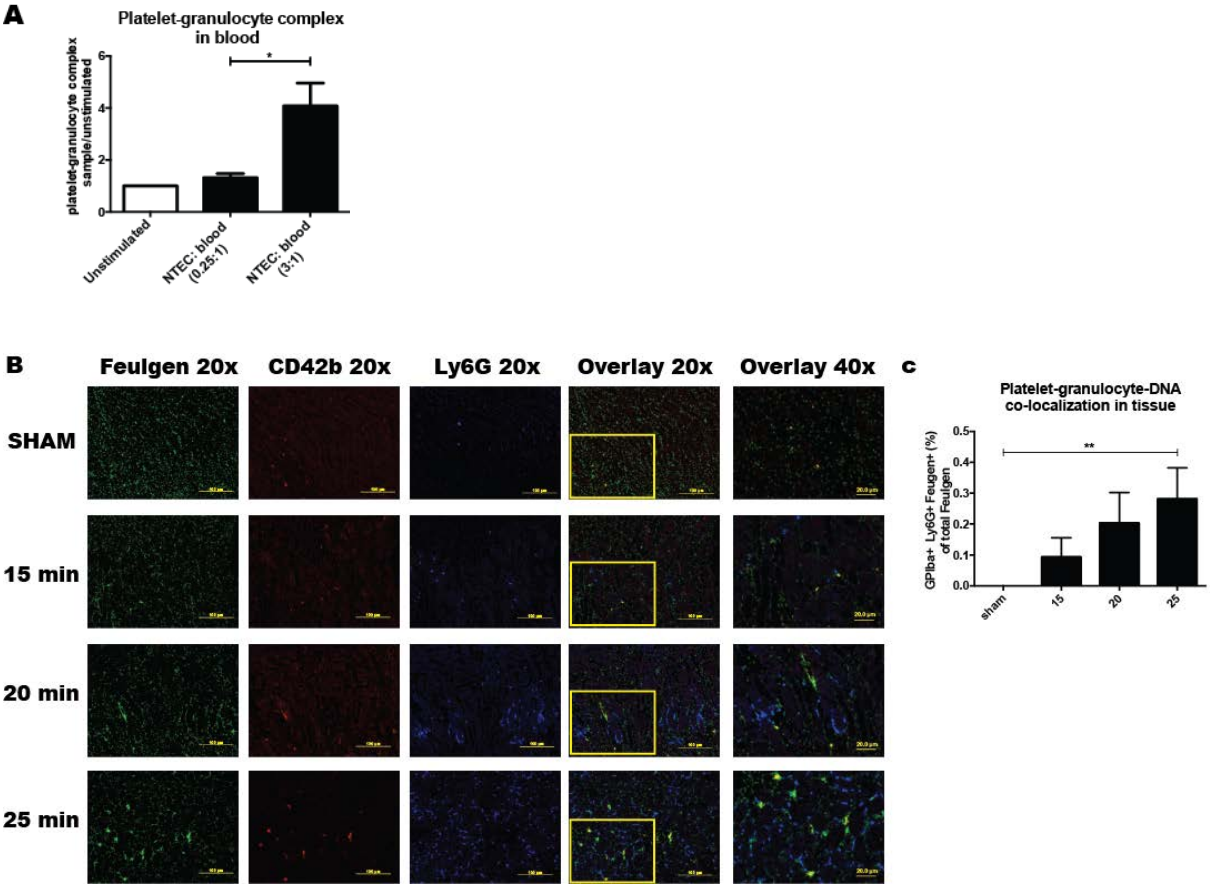
C
Plasma creatinine
umol/L
Sham
15
20
25
D
Plasma DNA
DNA concentration ng/mL
SHAM
15
20
25
E
Plasma PF4
PF4 pg/mL
SHAM
15
20
25
F
Feulgen 20x
CD42b 20x
Overlay 20x
Overlay 40x
SHAM
15 min
20 min
25 min
G
Platelet-DNA co-localization
CD42b within total Feulgen staining (%)
sham
15
20
25
H
Tubular necrosis/ DNA-PLT co-localization
r =0.68
P=0.0005
DNA-Platelet co-localization
Tubular Necrosis
I
Plasma urea
mmol/L
Vehicle
DNase I
J
Plasma creatinine
umol/L
Vehicle
DNase I
K
Tubular necrosis
Necrose scoring
Vehicle
DNAse I
L
KIM-1 mRNA
KIM-1/HPRT AU
Vehicle
DNase I
M
Renal MPO
mpo pg/ug protein
Vehicle
DNAse I
N
NGAL mRNA
NGAL/HPRT AU
Vehicle
DNase I

Figure 3 | ***Renal ischemia/reperfusion leads to increased DNA release and platelet activation.*** Renal dysfunction of wild-type mice with clamping times of the renal artery of 0, 15, 20, and 25 minutes and reperfusion for 24 hours as reflected by an increased tubular necrosis score (**a**) and levels of urea (**b**) and creatinine (**c**). **d**) DNA plasma levels in mice with clamped times of 0, 15, 20, and 25 minutes were measured using PicoGreen. (**e**) Plasma platelet factor 4 (PF4) levels in mice with clamping times of 0, 15, 20, and 25 minutes were measured with enzyme-linked immunosorbent assay. (**f**) Representative photographs with allocated fluorescent colors representing Feulgen (green), and GPIba (red) positive staining with colocalization (yellow) in renal tissue (overlay original magnification ×20 and ×40) in renal tissue sections of mice subjected to 0, 15, 20, or 25 minutes of renal clamping time. Original magnification, ×20 and ×40; bars = 100 μm or 20 μm. (**g**) Colocalization was measured and assessed as the percentage of GPIba staining positive within total Feulgen-positive staining. (**h**) Correlation was calculated between tubular necrosis and Feulgen/ GPIba colocalization. Plasma urea (**i**) and creatinine (**j**) in wild-type (WT) mice treated with vehicle (black columns) or DNase1 (gray column) 1 day after I/R. Score for histopathology of renal tubular damage using periodic acid–Schiff diastase–stained renal tissue sections (**k**) and measurement of kidney injury molecule-1 mRNA expression in WT treated with vehicle and WT treated with DNase1 before and after renal I/R (**l**). Measurement of renal MPO (**m**) and neutrophil gelatinase–associated lipocalin (nGAL) mRNA (**n**) in WT mice treated with vehicle (black columns) or DNase1 (gray column) 1 day after I/R. mRNA levels of neutrophil gelatinase–associated lipocalin (NGAL) and kidney injury molecule-1 (KIM-1) are expressed relatively to the housekeeping gene *HPRT*. Protein expression of myeloperoxidase (MPO) was corrected for total protein level in tissue. PLT, human platelets. Data are mean ± SEM of mice per group. **P* < 0.05, ***P* < 0.001, ****P* < 0.0001.

Renal tissue necrosis leads to increased platelet-granulocyte interaction that can result in neutrophil extracellular trap formation.

Activated platelets are known to interact with other cells such as granulocytes. This platelet-granulocyte interaction has been proven to be important in the activation and migration of granulocytes toward injured tissue.[14] Platelet-granulocyte interaction can lead to NET formation and plays a role during infectious diseases such as sepsis[28,29] and noninfectious diseases such as transfusion-related acute lung injury.[15] NETs are composed of mainly DNA, histones (e.g. citrulinated histone H3 [CitH3]) and granular content (e.g. MPO and elastase), which have been proven to be cytotoxic for tissue. Having shown that renal I/R injury promotes platelet activation, we hypothesized that renal injury stimulates platelet-granulocyte interaction, which possibly facilitates NET formation. By stimulating whole blood with NTEC supernatant, we demonstrated an increase of platelet-granulocyte complex formation ex vivo (Figure 4a). In addition, renal I/R resulted in a clamping time–dependent increase of platelet-DNA-granulocyte colocalization in renal tissue (Figure 4b and c). To verify that interaction of activated platelets with granulocytes can induce NETs, we stimulated granulocytes ex vivo with a high concentration of platelets activated with thrombin receptor–activating peptide. This resulted in a significant increase of NET production, reflected by released DNA, after 1 hour of incubation compared with stimulation with the RPMI medium control or a lower concentration of unstimulated platelets (Supplementary Figure S4).


A
Platelet-granulocyte complex in blood
platelet-granulocyte complex sample/unstimulated
*
Unstimulated
NTEC: blood (0.25:1)
NTEC: blood (3:1)
B
Feulgen 20x
CD42b 20x
Ly6G 20x
Overlay 20x
Overlay 40x
SHAM
15 min
20 min
25 min
c
Platelet-granulocyte-DNA co-localization in tissue
GPIba+ Ly6G+ Feugen+ (%) of total Feulgen
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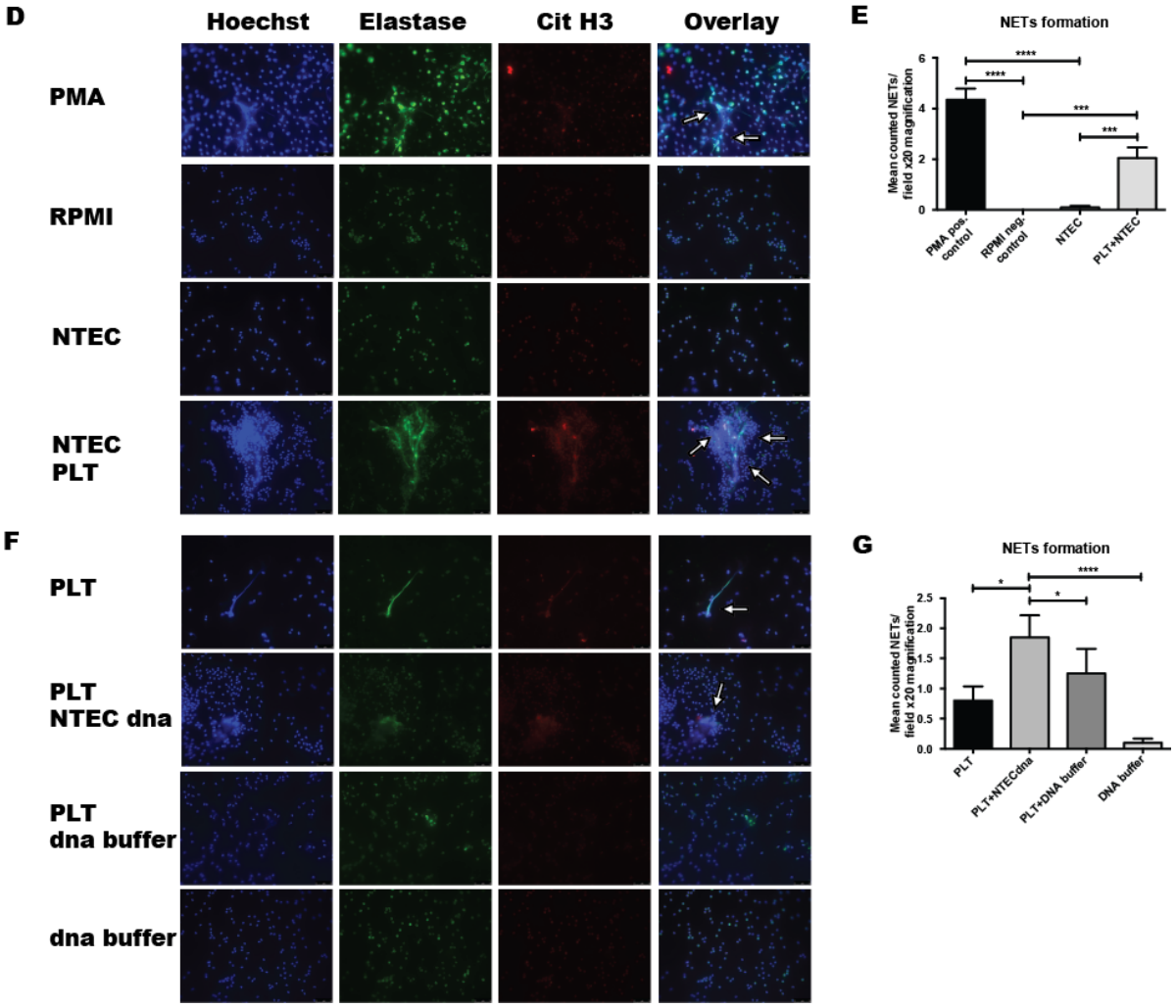
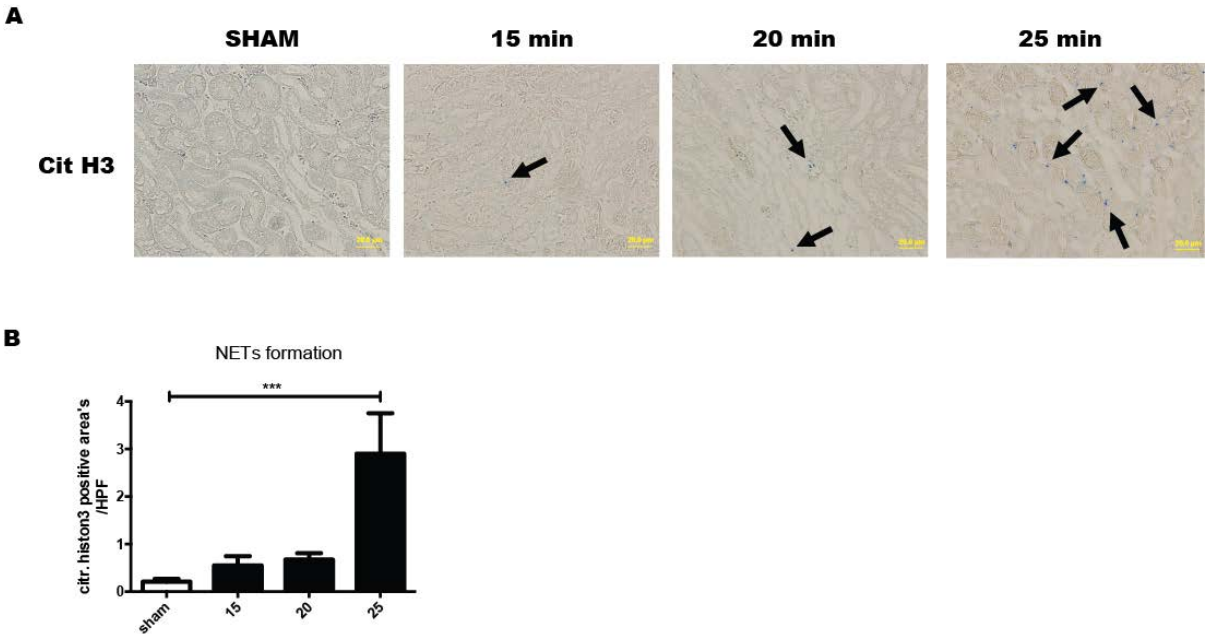


Figure 4 | ***Renal tissue necrosis leads to increased platelet-granulocyte interaction that can result in neutrophil extracellular trap formation.*** *Ex vivo* dose-response stimulation of human platelets in whole blood with 2 different volumes of supernatant derived from necrotic tubular epithelial cells (NTECs) (0.4×10^6 cells /ml), expressed as the ratio NTEC:blood. Platelet/granulocyte (CD61+/CCD66b+ high) complex formation of total leukocytes (CD45) gated is determined by flow cytometry analysis (**a**). Representative photographs with allocated fluorescent colors representing Feulgen- (green) and GPIba - (red) and lymphocyte antigen 6G (Ly6G)- (blue) positive staining with colocalization (yellow) in renal tissue sections (overlay original magnification ×20 and ×40) of mice subjected to 0-, 15-, 20-, or 25-minute renal clamping times; original magnification ×20 and ×40, bar = 100 μm or 20 μm (**b**). Colocalization was measured and assessed as the percentage of GPIba and Ly6G staining positive of the total Feulgen positive staining (**c**). Representative images (original magnification ×20, bars = 50 μm) from direct immunofluorescence staining of DNA (blue), elastase (green), and citrulinated histone 3 (red) from human granulocytes treated with phorbol myristate acetate (PMA) 100 nM (positive control), RPMI medium (negative control), necrotic tubular epithelial cells (NTECs), or human platelets (PLTs) + NTECs, with white arrows indicating neutrophil extracellular trap (NETs) (**d**) and counted in 20 randomly selected ×20 magnification fields (**e**). Representative images (original magnification ×20, bars = 50 μm) from direct immunofluorescence staining of DNA (blue), elastase (green), and citrulinated histone3 (CitH3) (red) from human granulocytes treated with PLTs, PLTs + NTEC DNA, PLTs + DNA buffer, or DNA buffer only (**f**), and counted in 20 randomly selected fields (original magnification ×20) (**g**). AE is DNA buffer. Data are mean ± SEM of mice per group. **P* < 0.05, ***P* < 0.001, ****P* < 0.0001, *****P* < 0.00001.

To test whether incubation of granulocytes with platelets stimulated with NTEC supernatant results in the formation of NETs, we isolated granulocytes from healthy volunteers and visualized NET formation after incubation of granulocytes with NTEC-stimulated platelets. PMA–treated granulocytes were used as a positive control, and RPMI medium–treated granulocytes were used as a negative control for NET formation. Stimulation of granulocytes with platelets in combination with NTEC resulted in significantly more NET formation compared with stimulation of granulocytes with NTEC only (Figure 4d and e). In addition, stimulating granulocytes with platelets in combination with DNA, isolated from NTEC, resulted in significantly elevated NET formation (Figure 4f and g).

Ischemia/reperfusion leads to neutrophil extracellular trap formation in renal tissue that is decreased by clopidogrel treatment.

To determine whether NETs are formed in vivo after renal I/R, we stained renal tissue for CitH3 and granulocytes. CitH3 has been proven to be a specific marker for NET formation. [30,31] CitH3 staining revealed the abundant presence of NETs in the corticomedullary region after renal I/R with 25 minutes of ischemia, which was significantly more compared with sham (Figure 5a and b).



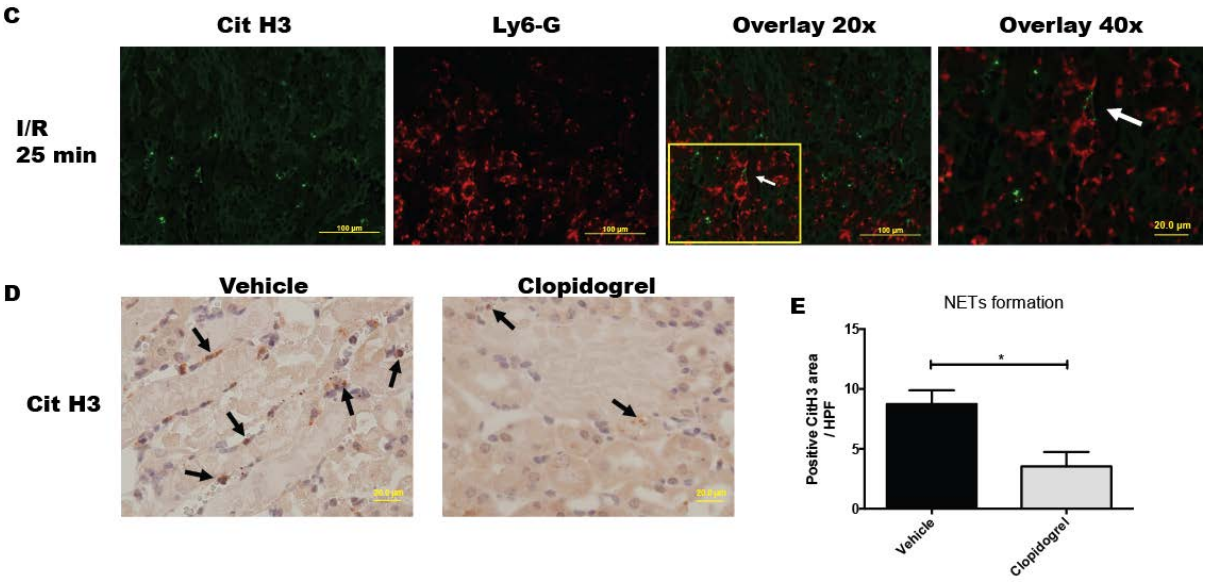


Figure 5 | Renal ischemic reperfusion leads to neutrophil extracellular trap formation in tissue that decrease upon platelet activation inhibition. Representative photographs of citrullinated histone H3 (CitH3) staining in kidneys from wild-type (WT) mice subjected to renal I/R with 0, 15, 20, and 25 minutes of ischemia, with black arrows indicating neutrophil extracellular traps (NETs) (**a**), counted in at least 10 randomly selected high-power fields in the corticomedullary region (**b**). Original magnification ×40, bars = 20 μm. Representative photographs with allocated fluorescent colors representing CitH3 (green) and lymphocyte antigen 6G (Ly6G) (red) positive staining with colocalization, indicated by white arrows, in renal tissue sections (overlay ×20 and ×40) of mice subjected to 25 minutes of renal clamping time, original magnification ×20 and ×40, bars = 100 μm and 20 μm (**c**). Representative photographs of CitH3 staining in kidneys from WT mice treated with vehicle compared with WT mice treated with clopidogrel subjected to renal I/R, with black arrows indicating NETs (**d**), counted in at least 10 randomly selected high-power fields in the corticomedullary region (**e**). Original magnification ×40, bars = 20 μm. Data are mean ± SEM of mice per group. HPF, high-power field. **P* < 0.05, ****P* < 0.0001.

Double staining of granulocytes and CitH3 demonstrated that CitH3 colocalizes with granulocytes in the corticomedullary region, confirming granulocyte-specific NETosis (Figure 5c). To study the effect of platelet inhibition on NET formation, we performed CitH3 staining of kidney sections of mice treated with vehicle or clopidogrel and subjected to renal I/R (Figure 5d). Quantification of CitH3-positive areas revealed a significant reduction of NET formation in mice treated with clopidogrel compared with mice treated with vehicle (Figure 5e), indicating that platelet inhibition decreases NET formation. In contrast, mice treated with DNase1 did not show less NET formation compared with mice treated with vehicle (Supplementary Figure S5).

Neutrophil extracellular traps stimulate platelet activation.

Because NETs were formed during renal I/R, we hypothesized that in addition to extracellular DNA derived from cell necrosis, NETs could also be a source of extracellular DNA that could activate platelets. Incubating washed platelets with preformed NETs indeed resulted in a

significant increase of platelet activation, as reflected by PF4 production, indicating that NETs can stimulate platelet activation (Figure 6).

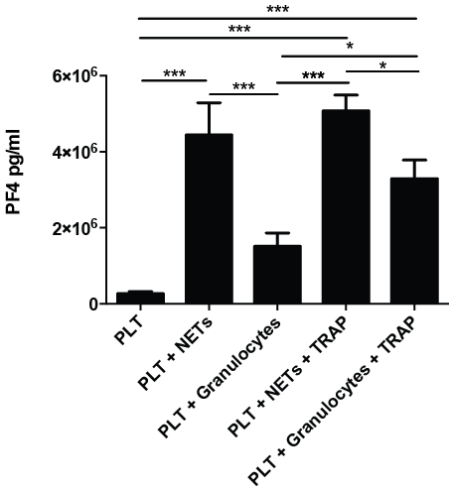


Figure 6 | Neutrophil extracellular traps stimulate platelet activation. Human platelet factor 4 (PF4) measurement in pg/ml after stimulation of human platelets (PLTs) with neutrophil extracellular traps NETs or granulocytes with or without thrombin receptor–activating peptide (TRAP). Data are mean ± SEM of mice per group. *$P < 0.05$, ***$P < 0.0001$.

The presence of a vicious triad linking renal cell necrosis, activated platelets, and neutrophil extracellular traps in the pathology of acute kidney injury. Taken together, we propose a mechanism in which extracellular DNA derived from necrotic renal cells can activate platelets, which stimulates platelet-granulocyte interaction and NET formation. Vice versa, the formation of NETs can activate platelets and is cytotoxic to cells, leading to more cell necrosis.[32,33] This detrimental vicious triad contributes to the pathology of acute renal injury and explains how platelet activation inhibition ameliorates AKI after renal I/R (Figure 7a).

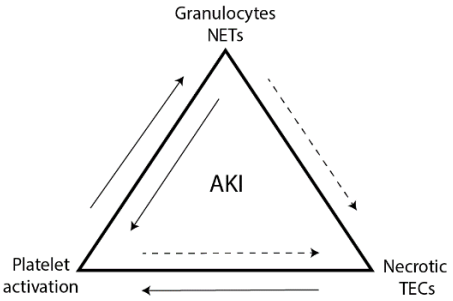


Figure 7 | The presence of a vicious triad linking renal cell necrosis, activated platelets, and neutrophil extracellular traps in the pathology of acute kidney injury. Proposed mechanism: a vicious triad including

neutrophil extracellular traps (NETs), necrotic tubular epithelial cells (TECs), and activated platelets in the kidney form a driving force in the pathophysiology of AKI.

Discussion

In renal I/R injury, inflammatory and coagulation processes contribute to tubular injury and subsequent renal dysfunction.[34] Platelets play a central role in inflammatory and coagulation processes and have been linked to the pathology of AKI. In a mouse model of AKI, clopidogrel treatment was shown to ameliorate renal I/R injury.[21] Furthermore, an observational cohort study on patients undergoing cardiac surgery demonstrated that preoperative use of aspirin is associated with a significant decrease in the risk of the development of I/R-induced acute renal failure, which supports a role for platelets in the pathophysiology.[35] To date, what triggers platelet activation in the I/R context is partly unknown. In this study, we aimed to elucidate what drives platelet activation during renal I/R and how platelet activation ultimately plays a role in the pathology of renal I/R injury. Our main findings are that platelet activation occurs in contact with necrotic cell casts in vivo and that platelet activation can be triggered by the release of extracellular DNA derived from NTECs, leading to platelet-granulocyte interaction. Treating mice with the platelet activation inhibitor clopidogrel resulted in less renal injury and granulocyte influx, indicating a direct effect of platelet activation on renal I/R–induced cell injury and granulocyte tissue migration.

Ex vivo, we demonstrated that co-incubation of granulocytes with platelets stimulated with NTEC-derived DNA results in the accumulation of NETs, which have been proven to be harmful for renal tissue.[32,33] In accordance, upon renal I/R, we found the presence of NETs, which were decreased in mice treated with clopidogrel, indicating a link between platelet activation and NET formation in vivo. These results indicate that platelet activation in the pathology of AKI can be triggered by extracellular DNA derived from injured renal cells, triggering platelet-granulocyte interaction and facilitating NET formation, which could aggravate renal injury during renal I/R.

Renal tubular cell necrosis is one of the main features followed by renal I/R.[34] Necrosis is characterized by loss of the integrity of the cell membrane and exposure of intracellular components such as DNA, either free (genomic or mitochondrial DNA) or bound to histones (nucleosomes). Extracellular DNA has been proven to activate innate immune cells.[36]

Platelets express a number of innate immune receptors including a range of pattern recognition receptors via which they can participate in the innate immune response.[37] Taken together, the positive correlation between renal injury and platelet-DNA colocalization in tissue together with the increase of extracellular DNA and PF4 levels in circulation upon renal I/R and the fact that extracellular DNA derived from NTECs activates platelets ex vivo indicate that released extracellular DNA is probably, at least partly, responsible for platelet activation during renal I/R.

Extracellular DNA derived from necrosis can be cleared by so-called waste-management nucleases, such as DNase1. This enzyme, mostly synthesized by the parotid gland and intestines but also the kidney in both rodents and humans,[38-40] is secreted in the blood stream to clean up DNA released from necrotic cells.[41] Under normal circumstances, extracellular DNA from dead cells or bacteria is sufficiently cleared. However, when the rate of cell death exceeds the capacity of local clearance of dead cells and subsequent cell material (e.g. extracellular DNA), this can evoke immune responses and subsequent injury, as seen in DNase1 knockout mice in which a lupus-like phenotype spontaneously develops.[42] We found that treating mice with DNase1 does not decrease tubular cell necrosis, but it does reduce the loss of renal function at 1 day post-I/R. Whether this protection can be subscribed to plasma extracellular DNA clearing and subsequent prevention of platelet activation remains indefinite as both plasma DNA and PF4 levels did not differ between treatment groups.

In the past decade, it has been shown that upon activation, granulocytes can form NET-like structures containing DNA, histones, and granular proteins. This feature is classically linked to infectious diseases such as sepsis and serves as a weapon of the innate immune system to catch and kill circulating bacteria.[43] However, NETs are also found in sterile inflammation including transfusion-related acute lung injury or preeclampsia in which the presence of NETs may be a maladaptive response leading to tissue injury. Although the accumulation of granulocytes in the kidney during I/R injury has been demonstrated to be important in the pathogenesis of AKI,[44] the presence of NETs in renal tissue after renal I/R has not been shown to date. Using CitH3 staining as a marker for NET formation, we are the first to demonstrate NET formation specifically in the corticomedullary region of renal tissue upon I/R. This was seen after 20 minutes, but most prominently after 25 minutes of ischemia, indicating that the extent of NET formation in renal tissue depends on the duration of

ischemia and possibly the level of platelet-granulocyte interaction. Like plasma DNA and PF4 levels, granulocyte influx and NET formation upon renal I/R did not differ in the DNase1-treated group, suggesting that granulocyte activation leading to NET formation was not affected by DNase1 treatment. However, MPO, one of the cytotoxic components expressed on NETs, was significantly decreased in DNase1-treated group. This could be an indication of NET dismantling by DNase-mediated digestion, losing NET-associated MPO and thus part of NET toxicity and explaining the mild protective phenotype observed in the DNase1-treated group. Taken together, we conclude that DNase1 treatment only slightly ameliorates AKI by targeting NETs that are already formed, whereas direct platelet activation inhibition has a more profound protected effect on AKI by preventing granulocyte activation and the formation of NETs.

It is known that activated platelets express adhesive molecules (e.g. P-selectin through which they interact with other [immune] cells such as granulocytes). This platelet-granulocyte interaction has been proven to be important in the activation and migration of granulocytes to injured tissue.[14] In the past few years, it has been demonstrated that platelet-granulocyte interaction can lead to NET formation and plays a role during infectious diseases such as sepsis[28,29] and noninfectious diseases such as transfusion-related acute lung injury.[15] We also found that platelet inhibition with clopidogrel results in significantly less NET formation in the kidney after I/R.

In this study, we demonstrated that granulocytes co-incubated with platelets stimulated by DNA derived from NTEC form NETs *ex vivo*. Co-incubation with NTEC or platelets only resulted in significantly less NET formation, indicating that, in particular, platelets stimulated with NTEC-derived DNA play a role in the formation of NETs. The protective effect of platelet activation inhibition on renal I/R injury *in vivo* could therefore be the result of decreased platelet-mediated NET formation. Here we demonstrate that NET formation can cause platelet activation. These results suggest the existence of a triad comprising activated platelets, necrotic cells, and NETs that contribute to renal damage and inflammation during renal I/R. Antiplatelet therapy can interfere with this triad by inhibiting platelet-mediated NET formation.

In conclusion, our results demonstrate that platelet activation during renal I/R can be caused by extracellular DNA derived from renal cell necrosis. We also show for the first time NET

formation in renal tissue, which possibly forms another source of leaked extracellular DNA, stimulates platelets, and contributes to renal tubular necrosis. DNA derived from cell necrosis can activate platelets to stimulate NET formation, maintaining a vicious triad of platelets, NETs, and cell necrosis. Targeting either platelet activation or NETs may be a new therapeutic strategy to ameliorate the progression of AKI.

Acknowledgments

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References

1 Hsu, R. K., McCulloch, C. E., Dudley, R. A., Lo, L. J. & Hsu, C. Y. Temporal changes in incidence of dialysis-requiring AKI. *J Am Soc Nephrol* **24**, 37-42 (2013).

2 M. D. Mayer Brezis, M. D., and Seymour Rosen. Hypoxia of renal medulla - Its implications for disease *N. Engl. J. Med.* **vol. 332**, pp. 647–655 (1995).

3 Padanilam, B. J. Cell death induced by acute renal injury a perspective on the contributions of apoptosis and necrosis. *Am. J. Physiol. Renal Physiol.* **vol. 284**, pp. F608–627 (2003).

4 Allam, R. *et al.* Histones from dying renal cells aggravate kidney injury via TLR2 and TLR4. *J Am Soc Nephrol* **23**, 1375-1388 (2012).

5 Ohashi, K., Burkart, V., Flohe, S. & Kolb, H. Cutting Edge: Heat Shock Protein 60 Is a Putative Endogenous Ligand of the Toll-Like Receptor-4 Complex. *The Journal of Immunology* **164**, 558-561 (2000).

6 I. Gursel, M. G., H. Yamada. Bacterial DNA-Induced Immune Activation 1. *The Journal of Immunology*, 171:1393-1400 (2014).

7 Lapchak, P. H. *et al.* Platelet-associated CD40/CD154 mediates remote tissue damage after mesenteric ischemia/reperfusion injury. *PLoS One* **7**, e32260 (2012).

8 Seligmann, C., Prechtl, G., Kusus-Seligmann, M. & Daniel, W. G. A myocardial ischemia- and reperfusion-induced injury is mediated by reactive oxygen species released from blood platelets. *Platelets* **24**, 37-43 (2013).

9 Pak, S. *et al.* Platelet adhesion in the sinusoid caused hepatic injury by neutrophils after hepatic ischemia reperfusion. *Platelets* **21**, 282-288 (2010).

10 Andonegui, G. *et al.* Platelets express functional Toll-like receptor-4. *Blood* **106**, 2417-2423 (2005).

11 Shiraki, R. *et al.* Expression of Toll-like receptors on human platelets. *Thromb Res* **113**, 379-385 (2004).

12 Beaulieu, L. M. & Freedman, J. E. The role of inflammation in regulating platelet production and function: Toll-like receptors in platelets and megakaryocytes. *Thromb Res* **125**, 205-209 (2010).

13 Aslam, R. *et al.* Platelet Toll-like receptor expression modulates lipopolysaccharide-induced thrombocytopenia and tumor necrosis factor-alpha production in vivo. *Blood* **107**, 637-641 (2006).

14 Page, C. & Pitchford, S. Neutrophil and platelet complexes and their relevance to neutrophil recruitment and activation. *Int Immunopharmacol* **17**, 1176-1184 (2013).

15 Caudrillier, A. *et al.* Platelets induce neutrophil extracellular traps in transfusion-related acute lung injury. *J Clin Invest* **122**, 2661-2671 (2012).

16 Thomas, G. M. *et al.* Extracellular DNA traps are associated with the pathogenesis of TRALI in humans and mice. *Blood* **119**, 6335-6343 (2012).

17 Oklu, R., Albadawi, H., Jones, J. E., Yoo, H. J. & Watkins, M. T. Reduced hind limb ischemia-reperfusion injury in Toll-like receptor-4 mutant mice is associated with decreased neutrophil extracellular traps. *J Vasc Surg* **58**, 1627-1636 (2013).

18 de Boer, O. J. *et al.* Neutrophils, neutrophil extracellular traps and interleukin-17 associate with the organisation of thrombi in acute myocardial infarction. *Thromb Haemost* **109**, 290-297 (2013).
19 Brill, A. *et al.* Neutrophil extracellular traps promote deep vein thrombosis in mice. *J Thromb Haemost* **10**, 136-144 (2012).
20 Savchenko, A. S. *et al.* VWF-mediated leukocyte recruitment with chromatin decondensation by PAD4 increases myocardial ischemia/reperfusion injury in mice. *Blood* **123**, 141-148 (2014).
21 Hu, H. *et al.* Clopidogrel protects from cell apoptosis and oxidative damage in a mouse model of renal ischaemia-reperfusion injury. *J Pathol* **225**, 265-275 (2011).
22 Pulskens, W. P. *et al.* Toll-like receptor-4 coordinates the innate immune response of the kidney to renal ischemia/reperfusion injury. *PLoS One* **3**, e3596 (2008).
23 Brinkmann, V., Laube, B., Abu Abed, U., Goosmann, C. & Zychlinsky, A. Neutrophil extracellular traps: how to generate and visualize them. *J Vis Exp* (2010).
24 Stokman, G., Leemans, J. C., Claessen, N., Weening, J. J. & Florquin, S. Hematopoietic stem cell mobilization therapy accelerates recovery of renal function independent of stem cell contribution. *J Am Soc Nephrol* **16**, 1684-1692 (2005).
25 Iyer, S. S. *et al.* Necrotic cells trigger a sterile inflammatory response through the Nlrp3 inflammasome. *Proc Natl Acad Sci U S A* **106**, 20388-20393 (2009).
26 Semple, J. W. & Freedman, J. Platelets and innate immunity. *Cell Mol Life Sci* **67**, 499-511 (2010).
27 Rosin, D. L. & Okusa, M. D. Dangers within: DAMP responses to damage and cell death in kidney disease. *J Am Soc Nephrol* **22**, 416-425 (2011).
28 Clark, S. R. *et al.* Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. *Nat Med* **13**, 463-469 (2007).
29 McDonald, B., Urrutia, R., Yipp, B. G., Jenne, C. N. & Kubes, P. Intravascular neutrophil extracellular traps capture bacteria from the bloodstream during sepsis. *Cell Host Microbe* **12**, 324-333 (2012).
30 Wang Y, L. M., Stadler S, Correll S *et al.* Histone hypercitrullination mediates chromatin decondensation and neutrophil extracellular trap formation. *J Cell Biol* **184**, 205-213 (2009).
31 Leshner, M. *et al.* PAD4 mediated histone hypercitrullination induces heterochromatin decondensation and chromatin unfolding to form neutrophil extracellular trap-like structures. *Front Immunol* **3**, 307 (2012).
32 Villanueva, E. *et al.* Netting neutrophils induce endothelial damage, infiltrate tissues, and expose immunostimulatory molecules in systemic lupus erythematosus. *J Immunol* **187**, 538-552 (2011).
33 Saffarzadeh, M. *et al.* Neutrophil extracellular traps directly induce epithelial and endothelial cell death: a predominant role of histones. *PLoS One* **7**, e32366 (2012).
34 Bonventre, J. V. & Yang, L. Cellular pathophysiology of ischemic acute kidney injury. *J Clin Invest* **121**, 4210-4221 (2011).

35 Cao, L., Silvestry, S., Zhao, N., Diehl, J. & Sun, J. Effects of preoperative aspirin on cardiocerebral and renal complications in non-emergent cardiac surgery patients: a sub-group and cohort study. *PLoS One* **7**, e30094 (2012).

36 Sirois, C. M. *et al.* RAGE is a nucleic acid receptor that promotes inflammatory responses to DNA. *J Exp Med* **210**, 2447-2463 (2013).

37 Duerschmied, D., Bode, C. & Ahrens, I. Immune functions of platelets. *Thromb Haemost* **112**, 678-691 (2014).

38 Napireo, M., Ricken, D. & Eulitz, D. Expression pattern of the deoxyribonuclease 1 gene lessons from the Dnase1 knockout mouse. *Biochem. J.* **380**, 929–937 (2004).

39 Lacks, S. A. Deoxyribonuclease I in Mammalian Tissues. *Journal of biological chemistry* **256**, 2644-2648 (1981).

40 Nadano, D., Yasuda, T. & Kishi, K. Measurement of Deoxyribonuclease I Activity in Human Tissues and Body Fluids by a Single Radial Enzyme-Diffusion Method. *Clin. Chem*, 448-452 (1993).

41 Napirei, M., Wulf, S. & Mannherz, H. G. Chromatin breakdown during necrosis by serum Dnase1 and the plasminogen system. *Arthritis Rheum* **50**, 1873-1883 (2004).

42 Walport, M. J. Lupus, DNase and defective disposal of cellular debris. *Nature America inc.* (2000).

43 Brinkmann, V. *et al.* Neutrophil extracellular traps kill bacteria. *Science* **303**, 1532-1535 (2004).

44 Awad, A. S. *et al.* Compartmentalization of neutrophils in the kidney and lung following acute ischemic kidney injury. *Kidney Int* **75**, 689-698 (2009).

Supplementary figures

Figure S1

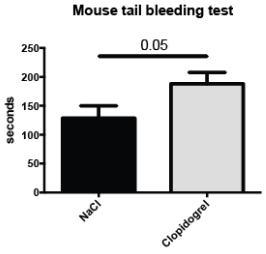


Figure S2

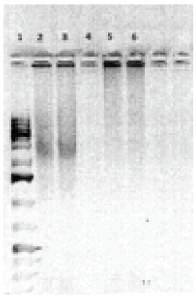


Figure S3

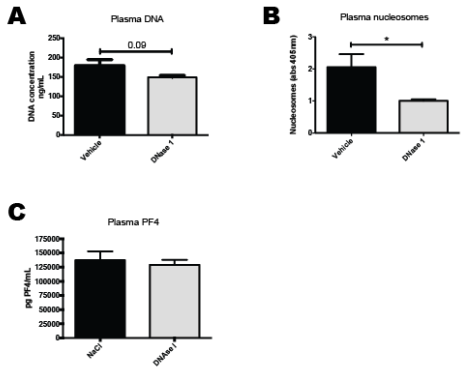


Figure S4

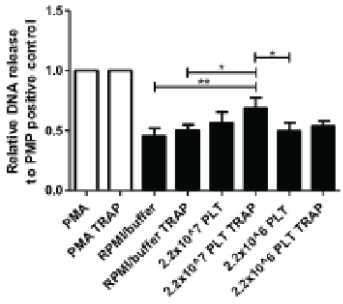
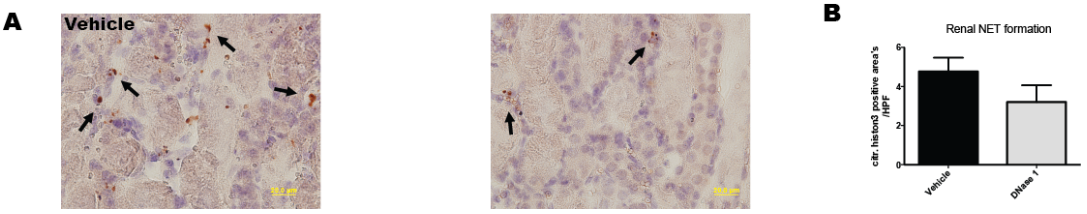


Figure S5



Supplementary figures. Figure S1. Tail bleeding test. Tail bleeding test performed on mice treated with clopidogrel (5 mg/kg per day) or vehicle (0.9% NaCl). Data are mean ± SEM of mice per group. $P = 0.05$. **Figure S2.** Extracellular DNA released from proximal NTECs. DNA detection in NTEC supernatant and NTEC supernatant incubated with 8 U/ml DNase 1. Lane 1 = 1-kb ladder, lanes 2 and 3 = NTECs, lanes 5 and 6 = NTECS incubated with 8/U ml DNase 1. NETCs, necrotic tubular epithelial cells. **Figure S3.** Plasma DNA, nucleosomes, and PF4 levels upon renal I/R in mice treated with DNase 1 or vehicle. DNA levels (**A**), nucleosome levels (**B**), and PF4 concentration (**C**) measured in plasma of mice subjected to renal I/R and treated with DNase1 or vehicle. $*P < 0.05$. I/R, ischemia/reperfusion; PF4, platelet factor 4. **Figure S4.** Activated platelets induce NETs. *Ex vivo* NET measurement expressed as concentration of released DNA after stimulation with human control buffer or human platelets (2.2×10^7 or 2.2×10^6) with or without thrombin receptor activating peptide stimulation (black bars), relative to positive control of phorbol myristate acetate–stimulated granulocytes with or without thrombin receptor–activating peptide stimulation (white bars). Data are mean ± SEM of mice per group. $*P < 0.05$, $**P < 0.001$. NETs, neutrophil extracellular traps; PLT, platelets; PMA, phorbol myristate acetate; TRAP, thrombin receptor activating peptide. **Figure S5.** Effect of DNase 1 treatment on NET formation in renal tissue upon renal I/R. Representative photographs of citrullinated histone H3 staining in kidneys from wild-type mice subjected to renal I/R and treated with vehicle (0.9% NaCl) or DNase1, with black arrows pointing to NETs (**A**), counted in at least 10 randomly selected high-power fields in the corticomedullary region (**B**). Original magnification ×40; bars = 20 μm. Citr., citrullinated; HPF, high-power field; IR, ischemia/reperfusion; NET, neutrophil extracellular trap.

Chapter 5

No difference in renal injury and fibrosis between Wild-type and NOD1/NOD2 double knockout mice with chronic kidney disease induced by ureteral obstruction

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Abstract

Background: Chronic kidney disease (CKD) is characterized by sustained tissue damage and ongoingtubulo-interstitial inflammation and fibrosis. Pattern recognition receptors (PRRs) including Toll-like receptors (TLRs) and NOD-like receptors (NLRs) can sense endogenous ligands released upon tissue damage, leading to sterile inflammation and eventually irreversible kidney disease. It is known that NOD1 and NOD2 contribute to the pathogenesis of various inflammatory diseases, including acute kidney injury. However their role in chronic kidney disease is largely unknown. The aim of this study was therefore to investigate the contribution of NOD1 and NOD2 in renal interstitial fibrosis and obstructive nephropathy.

Methods: To do so, we performed unilateral ureteral obstruction (UUO) in wild type (WT) and NOD1/NOD2 double deficient (DKO) mice and analysed renal damage, fibrosis and inflammation. Data were analysed using the non-parametric Mann-Whitney U-test.

Results: Minor changes in inflammatory response were observed in NOD1/2 DKO mice, while no effects were observed on renal injury and the development of fibrosis.

Conclusion: No difference in renal injury and fibrosis between WT and NOD1/NOD2 DKO mice following obstructive nephropathy induced by ureteral obstruction.

Key words: Pattern recognition receptors, NOD1, NOD2, renal fibrosis, obstructive nephropathy

Background

NOD1 and NOD2 are members of the cytoplasmic PRR family of NLRs. PRRs are important in mediating a rapid response to pathogens via recognition of several highly conserved pathogen- associated molecular patterns (PAMP). In addition to PAMPs various (endogenous) damage-associated molecular patterns (DAMP) or stress signals have been identified that can initiate sterile inflammation [1]. Upon renal injury DAMPs are released such as biglycan, high-mobility group box 1 (HMGB1), and hyaluronic acid that can signal via TLRs and NLRs [2-5]. NOD1 and NOD2 detect specific substructures from bacterial peptidoglycan (PGN). NOD1 senses $Gram^-$-derived PGN containing Tri-DAP [6, 7], while NOD2 senses $Gram^-$- and $Gram^+$-derived PGN containing MDP [8, 9]. In line, we found that NOD1/2 are involved in the development of acute renal disease during septic shock induced by bacterial components [10]. In addition to bacterial structures, Sabbah *et al* reported the activation of NOD2 by single-stranded RNA viruses [11]. Recently, activation of NOD1 and NOD2 by the non-pathogenic derived cell permeable small molecule DMXAA was reported [12]. As far as we know, no endogenous DAMPs for NOD1 and NOD2 are described. However, based on their structural and functional similarities with other NLR family members and TLRs it could be speculated that NOD1 and NOD2 are also activated by currently unknown endogenous ligands. In line with this reasoning, Shigeoka et al. showed that NOD1 and NOD2 participate in acute renal ischemia reperfusion injury (IRI), suggesting that these receptors are able to respond to endogenous ligands released upon IRI [13]. NOD1 is widely expressed in many cell types and organs including the tubular epithelial cells (TEC) in human and mouse kidney [13-16]. NOD2 is expressed on murine TECs, mesangial cells, podocytes and on human TECs and glomerular endothelial cells [13, 17, 18]. Given the expression of NOD1 and NOD2 in the kidney and more specific in TEC and the fact that NOD1/2 play a deleterious role in acute kidney disease could suggest that these PRRs contribute to the pathogenesis of chronic renal damage as well. PRRs like NLRP3 and TLR4 have already been shown to play a role in obstructive nephropathy [19-21]. However, nothing is known about the role of NOD1 and NOD2 in inflammation and fibrosis during obstructive nephropathy. In the present study we therefore investigated the role of NOD1 and NOD2 in a model of obstructive nephropathy induced by ureteral obstruction.

Methods

Mice

Pathogen-free 8- to 12-week old female C57Bl/6 WT mice were purchased from Janvier (Le Genest, France). NOD1/NOD2 DKO mice were generated from NOD1 and NOD2 knockout mice and backcrossed to C57Bl/6 background at least 10 generations as described before [22]. Previously, we have characterized the DKO mice phenotypically and this revealed that except for lower liver weight in NOD1/2 DKO mice, there were no differences in body/organ weight, leukocyte count/composition and plasma biochemical markers between both strains [10]. NOD1/2 DKO mice were bred in the animal facility of the Academic Medical Center in Amsterdam, The Netherlands. Age- and sex-matched mice were used in all experiments. The animal and Use Committee of the University of Amsterdam approved all experiments.

Unilateral ureter obstruction

Mice (N=9/group) received a pre-operative dose of analgesia (0.15 mg/kg buprenorfine, subcutaneously) and were anesthetized by inhalation of 3% isoflurane, 0.2% N_2O and 2% O_2 during the surgical procedure. The right ureter was permanently ligated via a ventral approach using 6-0 silk (Tyco, Gosport, UK). The ureter was ligated at the height of the lower part of the kidney. Mice were sacrificed 3, 7 and 14 days after surgery via a heart puncture (blood collection) followed by cervical dislocation under general anaesthesia. Kidneys were snap frozen in liquid nitrogen and stored at -80^{o}C or fixed in 10% formalin o/n prior to further processing. Contralateral non-obstructed kidneys served as control.

Quantitative real-time RT-PCR

Total RNA was extracted from kidney using the TRIzol® reagent (Invitrogen, Breda, The Netherlands) and converted to cDNA. Quantitative real-time RT-PCR was performed on a LightCycler® 480 System (Roche, Mijdrecht, The Netherlands) using LightCycler® 480 SYBR Green I Master mix (Roche). Specific gene expression was normalized towards the reference gene TATA box binding protein (TBP). Primer sequences are as follows: NOD1 forward 5'-tcagactcagcgtcaaccag-3' and reverse 5'-taaacccaggaacgtcacga-3', NOD2 forward 5'-gggagatgttggagtggaac-3' and reverse 5'-agcgaagagcacactcaacc-3', and TBP forward 5'-ggagaatcatggaccagaaca-3' and reverse 5'-gatgggaattccaggagtca-3'.

Histology and immunohistochemistry

Formalin-fixed tissue was embedded in paraffin using standard procedures. Four-µm thick sections were cut and used for all stainings. For examining renal histology, sections were stained with periodic acid-Schiff reagents after diastase digestion (PasD). Injury to tubules was assessed (blinded) by determining the percentage of affected tubules per 10 fields (magnification x400) semi-quantitatively on a scale from 0 to 4 (0 = 0%, 1 = <25%, 2 = 25-50%, 3 = 50-75%, and 4 = >75%) according to the following criteria: tubular dilatation, epithelial simplification, and interstitial expansion in the cortex. For immunohistochemistry, sections were stained with FITC-labelled anti-mouse Ly-6G (Pharmingen, BD Biosciences, Alphen a/d Rijn, The Netherlands), rat anti-mouse F4/80 (Serotec, Oxford, UK), rabbit anti-mouse active caspase-3 (Cell Signaling Technology, Beverly, MA, USA), rabbit anti-human Ki67 (Neomarkers, Fremont, CA, USA), rabbit polyclonal to collagen type I (GeneTex, Irvine, CA, USA), or mouse anti-human αSMA (DAKO, Heverlee, Belgium) to detect granulocytes, macrophages, apoptosis, proliferation, collagen type I, and myofibroblasts respectively. The number of Ly6 positive cells and the number of caspase-3 and Ki67 positive TEC was counted in 10 non-overlapping fields (magnification x400) in a blinded manner. The percentage of positive staining for F4/80, collagen type I, total collagen and αSMA in obstructed kidneys was analysed using a computer-assisted digital analysis program (Image Pro-plus®, Media Cybernetics). At least 15 visual fields were sampled from the cortex of each kidney (magnification x20).

Statistical analyses

All statistical analyses were performed using GraphPad Prism 5 software (San Diego, CA, USA). Data were analysed using the non-parametric Mann-Whitney U-test. Results are expressed as mean ± standard error of the mean (SEM). $P<0.05$ was considered statistically significant.

Results

The role of NOD1 and NOD2 in CKD was investigated using the mouse model UUO. First we analysed the expression of NOD1 and NOD2 mRNA in WT kidney at several time points after UUO. We found expression of both transcripts in the kidney (figure 1), which were not altered during the development of obstructive nephropathy.

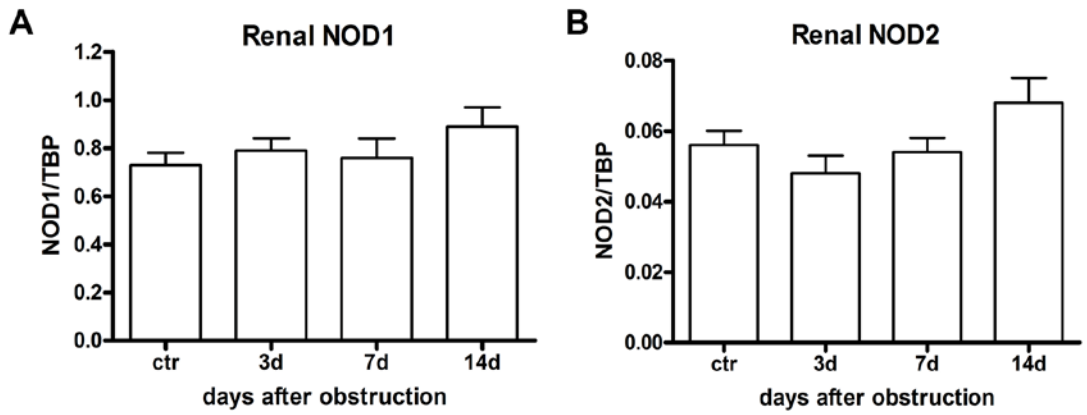


Figure 1: **Renal expression of NOD1 (A) and NOD2 (B) after 0, 3, 7, and 14 days following obstruction.** Gene expression was normalized towards the reference gene TBP. Data are expressed as mean ± SEM. N=9/group.

Tubular injury, as assessed by scoring PAS-D-stained kidney sections, increased markedly after UUO with a similar degree of damage in WT and NOD1/2 DKO at all time points examined (figure 2A, C). In line with the injury score, there were no differences in KIM-1 expression between the WT and the KO mice at all time points (figure 2B). KIM-1 was declined at day 14 of UUO possibly as an adaptation to prevent or slow down KIM-1 mediated chronic inflammation and renal fibrosis, as described previously [23].

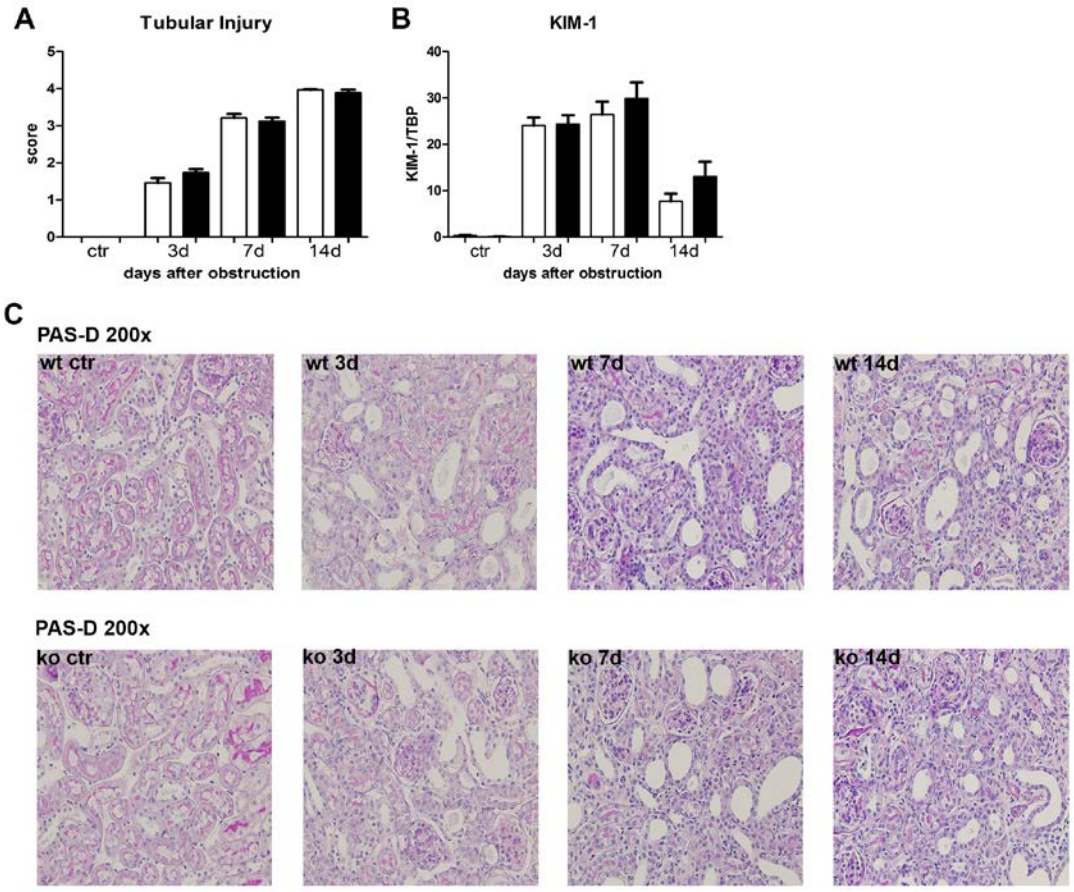


***Figure 2*: Renal injury in WT (white bars) and NOD1/2 DKO (black bars) mice after 0, 3, 7, and 14 days following obstruction.** Renal damage was evaluated by blinded scoring of the necrotic tubules in PAS-D-stained sections (A, C) and by measuring the mRNA expression of KIM-1 (B) in total kidney homogenates. Data are expressed as mean ± SEM. Results were analysed with the non-parametric two-tailed Mann-Whitney U-test. *$*P<0.05$*. N=9/group.

Tubulointerstitial injury in obstructed kidneys can result in an imbalance between TEC apoptosis and proliferation. Apoptosis and proliferation of TEC was increased at all investigated time points after obstruction (figure 3). However, no difference between WT and NOD1/2 DKO mice was observed.

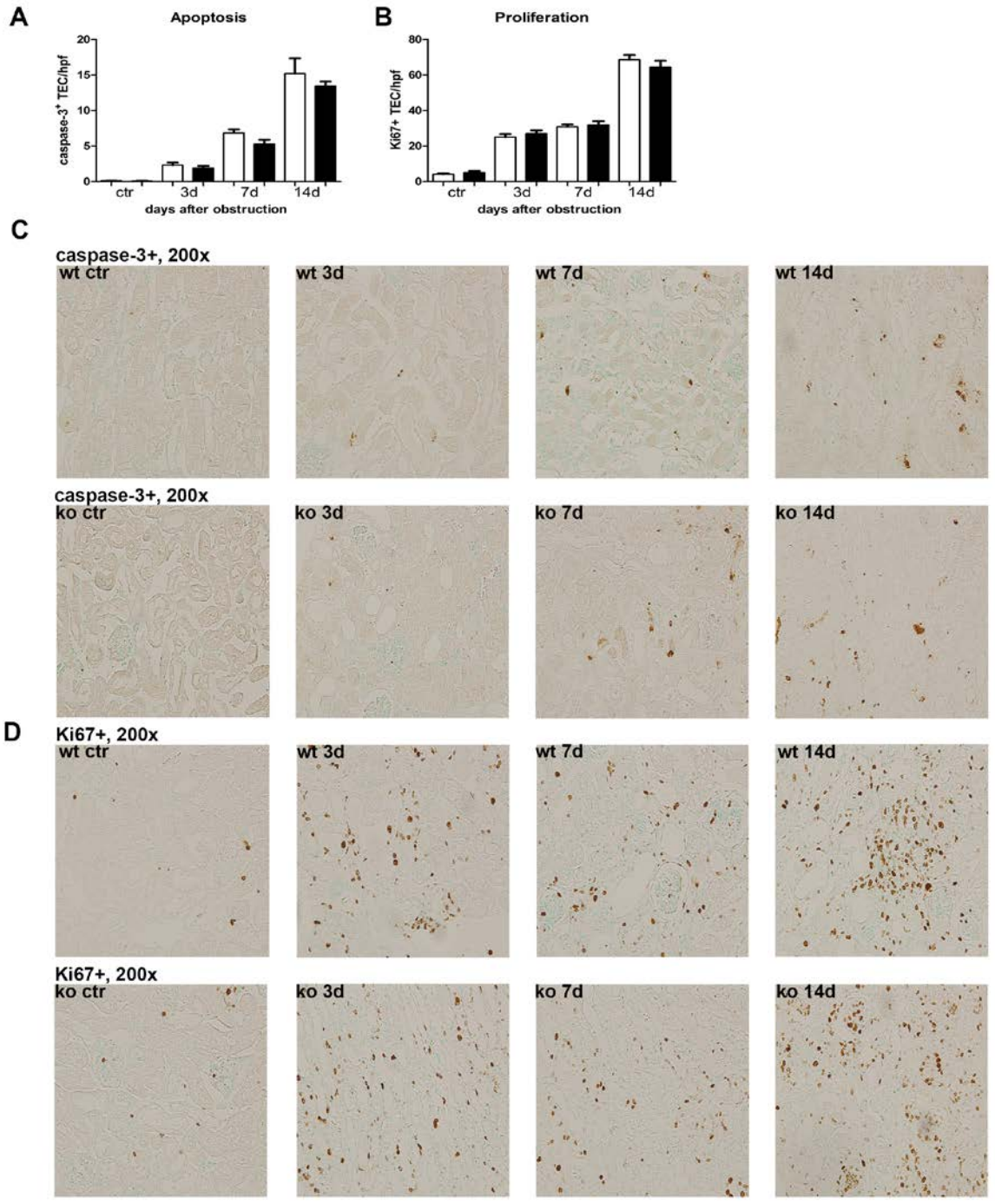


Figure 3: **Apoptosis and proliferation of TECs in WT (white bars) and NOD1/2 DKO (black bars) mice after 0, 3, 7, and 14 days following obstruction.** The amount of apoptotic TEC was determined by scoring caspase-3+ TEC (A, C) and the amount of proliferating TEC (B, D) was determined by scoring Ki67+ TEC. Data are expressed as mean ± SEM. N=9/group.

Fibrosis was determined by collagen type I (figure 4A) and total collagen deposition (supplementary figure 2). In both WT and NOD1/2 DKO obstructed kidneys fibrosis increased progressively, however no difference between the WT and NOD1/2 DKO mice was observed. Next we analysed the amount of myofibroblasts by αSMA immunohistochemistry (figure 4B). In line with tubular injury and fibrosis, the amount of myofibroblasts increased after UUO. Although myofibroblast accumulation was lower in NOD1/2 DKO mice 3 days following ureteral obstruction compared to WT mice, no differences were found after 7 and 14 days (figure 4B). One of the early events in progressive renal injury is the induction of chemokines and the subsequent recruitment of inflammatory cells.

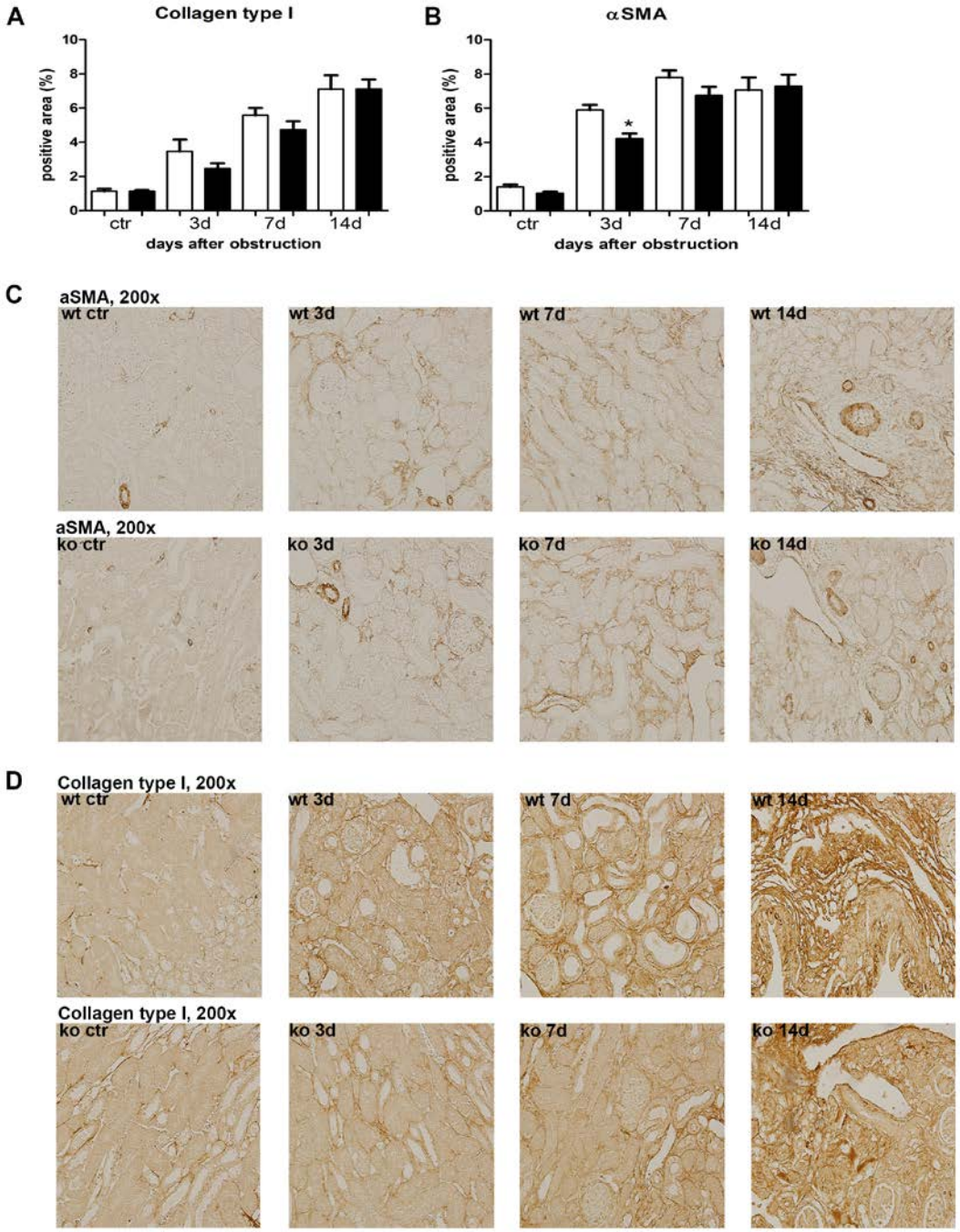
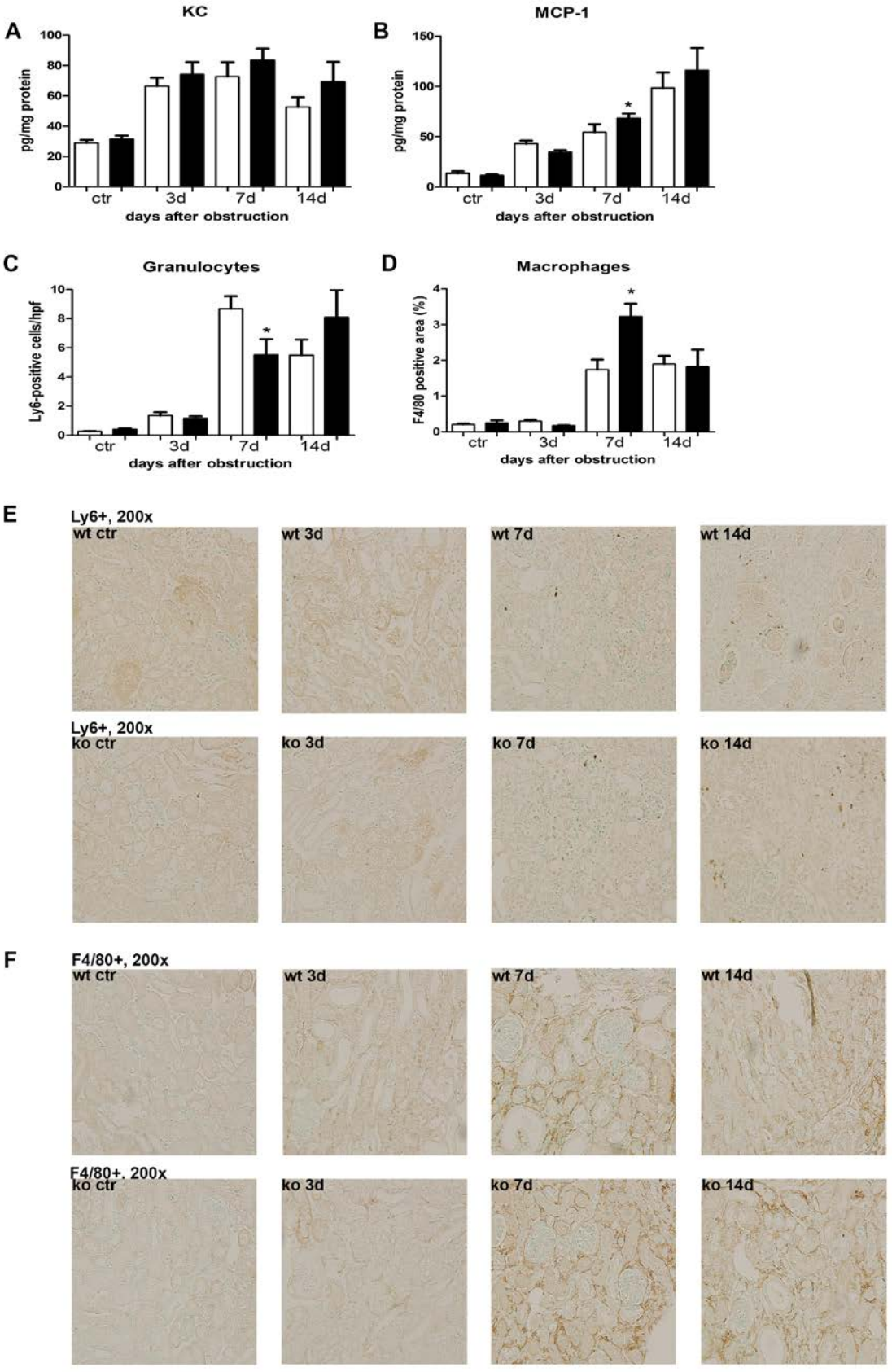


Figure 4: **Renal fibrosis in WT (white bars) and NOD1/2 DKO (black bars) mice after 0, 3, 7, and 14 days following obstruction.** Collagen type I deposition in kidneys was determined by Collagen type I staining and digitally analysed (A, D). Myofibroblast accumulation in kidneys was assessed by αSMA staining and digitally analysed (B, C). Data are expressed as mean ± SEM. Results were analysed with the non-parametric two-tailed Mann-Whitney U-test. *$P<0.05$. N=9/group.

The granulocyte chemoattractant KC (figure 5A) and the monocyte chemoattractant MCP-1 (figure 5B) increased significantly following obstruction in both WT and NOD1/2 DKO kidneys. Except for a slight but significant higher MCP-1 level in kidneys from NOD1/2 DKO mice compared with WT 7 days following obstruction, no difference in KC and MCP-1 levels were observed between the WT and NOD1/2 DKO mice. The influx of granulocytes (figure 5C, E) and accumulation of macrophages (figure 5D, F) increased in the obstructed WT and NOD1/2 DKO kidneys, yet there were no differences in these parameters between the WT and NOD1/2 DKO mice, 3 and 14 days post UUO. Seven days following obstruction there was a slight but significant decrease in granulocyte influx and a significant increase in macrophage accumulation in NOD1/2 DKO kidneys compared with WT kidneys.


A
KC
pg/mg protein
100
80
60
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ctr
3d
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days after obstruction
B
MCP-1
pg/mg protein
150
100
50
0
*
ctr
3d
7d
14d
days after obstruction
C
Granulocytes
Ly6-positive cells/hpf
10
8
6
4
2
0
*
ctr
3d
7d
14d
days after obstruction
D
Macrophages
F4/80 positive area (%)
4
3
2
1
0
*
ctr
3d
7d
14d
days after obstruction
E
Ly6+, 200x
wt ctr
wt 3d
wt 7d
wt 14d
Ly6+, 200x
ko ctr
ko 3d
ko 7d
ko 14d
F
F4/80+, 200x
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F4/80+, 200x
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ko 14d

***Figure 5*: Renal inflammation in WT (white bars) and NOD1/2 DKO (black bars) mice after 0, 3, 7, and 14 days following obstruction.** KC (A) and MCP-1 (B) were measured in total kidney homogenates with specific ELISAs. Influx of granulocytes was determined by scoring Ly6+ cells (C, E). Influx of macrophages was assessed by F4/80 staining which was digitally analysed (D, F). Data are expressed as mean ± SEM. Results were analysed with the non-parametric two-tailed Mann-Whitney U-test. *$P<0.05$. N=9/group.

To get more insight in the activation of the NF-kB signaling pathway, we measured the downstream cytokines IL-1b and TNF-α in total kidney homogenates. This revealed no significant differences between the WT and NOD1/2 DKO mice, expect for IL-1b day 3 (Supplementary figure 3). Together these results reveal that there are no or minor changes in the inflammatory response, renal damage and fibrosis following CKD induced by ureteral obstruction in NOD1/2 DKO mice compared with WT mice.

Discussion

To investigate the role of NOD1 and NOD2 in chronic renal inflammation, injury and fibrosis we subjected NOD1/2 DKO and WT mice at various time points to UUO. UUO initiates a sequence of events in the obstructed kidney, including interstitial inflammation and TEC death, ultimately leading to renal fibrosis which is the final common pathway for numerous forms of progressive renal disease. Recently, the role of the PRRs TLR2, TLR9, TLR4 and NLRP3 in progressive renal injury was investigated. Although TLR2 initiates the inflammatory response during obstructive nephropathy, it does not play a significant role in the development of renal progressive injury and fibrosis [3, 24]. Similarly, TLR9 was not involved in the pathogenesis of UUO [24]. On the other hand, TLR4 attenuates tubular damage and does contribute to renal fibrosis during obstructive nephropathy as demonstrated by an increased injury score and decreased collagen deposition in TLR4-deficient mice [20, 25]. Other work implied a central role for NLRP3 in renal inflammation, fibrosis and tubular damage at different phases of UUO [19, 21]. Apparently, different PRR members have unique response during obstructive nephropathy that lead to a profoundly different outcome of local injury and tubulointerstitial inflammation and fibrosis.

From our study we conclude that NOD1 and NOD2 do not play a significant role in the development of tubulointerstitial fibrosis and inflammation nor in the progression of renal damage after UUO-induced injury. No differences were observed between WT and NOD1/2

DKO obstructed kidneys regarding tubular injury score, apoptosis, proliferation and myofibroblast accumulation. A marginal effect of NOD1/2 deficiency could be detected in the inflammatory response during obstructive nephropathy. Slightly more MCP-1 and concomitant increased macrophage accumulation was observed in the NOD1/2 DKO kidney 7 days following obstruction, while granulocyte influx was lower at this time point. The majority of infiltrating leukocytes into the UUO-damaged kidney are macrophages, which produce cytokines responsible for tubular apoptosis and fibroblast proliferation and activation. However, enhanced macrophage accumulation did not affect the progression of renal fibrosis in NOD1/2 DKO mice. In another study on progressive kidney disease, namely diabetes, it was shown that NOD2 is upregulated and promoted the transcription of extracellular matrix genes and renal injury by inducing inflammation and podocyte insulin resistance [18]. Moreover, in sepsis- and ischemia-induced acute kidney disease models, NOD1/2 DKO mice were demonstrated to be protected against renal disease [10, 13]. Apparently, as it was the case for TLR2 [3, 26], NOD1 and NOD2 are involved in the initiation of inflammation but do not necessarily contribute to further renal damage and fibrosis. Considering that we found no difference between WT and NOD1/2 DKO mice in renal pathology, one could speculate that despite their structural and functional similarities with other PRRs, NOD1 and NOD2 are not activated by DAMPs that are released after UUO. This would be in line with current literature in which solely bacterial ligands and not DAMPs are described to activate NOD1 and NOD2. We anticipate that the difference in renal pathology between WT and NOD1/2DKO mice in the renal ischemia reperfusion injury model might be due to translocation of bacterial products across the leaky intestinal barrier that activate NOD1/2 resulting in inflammation-associated nephropathy. The phenomenon of intestinal barrier disruption is known to occur after renal ischemia reperfusion injury [27] but is not described for UUO.

This study however, has some limitations which have to be pointed out. By using double knockout mice we cannot rule out the possibility that either NOD1 or NOD2 play a different role in UUO and compensate each other. Alternatively, the function of NOD1 or NOD2 might be masked in our knockout model by redundancy or compensatory mechanism. Our experiments tested moreover only unilateral ureteral obstruction with its own advantages and limitations [28] and no other animal models of chronic kidney disease and fibrosis. Other possible roles of NOD1/NOD2 in these disorders that may be activated under different circumstances remain therefore to be tested.

Taken together, our data do not show a functional role for NOD1/2 in kidney injury and fibrosis following chronic kidney disease induced by ureteral obstruction and suggest that similar to infection, different forms of sterile kidney disease will be sensed by different PRRs triggering different signalling pathways which culminate in different kidney disease outcomes.

Conclusion

Together these results reveal that there are no or minor changes in the inflammatory response, renal damage and fibrosis following obstructive nephropathy induced by ureteral obstruction in NOD1/2 DKO mice compared with WT mice.

Abbreviations

CKD: Chronic kidney disease; PRRs: Pattern recognition receptors; TLRs: Toll-like receptors; NLRs: NOD-like receptors; UUO: unilateral ureteral obstruction; WT: wild type; DKO: double deficient; PAMP: pathogen associated molecular patterns; DAMP: danger associated molecular patterns; HMGB1: high-mobility group box 1; PGN: peptidoglycan; IRI: ischemia reperfusion injury; TEC: tubular epithelial cell; SEM: standard error of the mean .

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Ethics approval and consent to participate

All animal experiments were approved by the Animal and Use Committee of the University of Amsterdam. C57Bl/6 WT mice were purchased from Janvier (Le Genest, France). NOD1/NOD2 DKO were provided by Girardin's lab via a verbal consent between JCL and SEG.

References

1. Leemans, J.C., et al., *Pattern recognition receptors and the inflammasome in kidney disease.* Nat Rev Nephrol, 2014. **10**(7): p. 398-414.

2. Iyer, S.S., et al., *Necrotic cells trigger a sterile inflammatory response through the Nlrp3 inflammasome.* Proc Natl Acad Sci U S A, 2009. **106**(48): p. 20388-93.

3. Leemans, J.C., et al., *The role of Toll-like receptor 2 in inflammation and fibrosis during progressive renal injury.* PLoS One, 2009. **4**(5): p. e5704.

4. Schaefer, L., et al., *Absence of decorin adversely influences tubulointerstitial fibrosis of the obstructed kidney by enhanced apoptosis and increased inflammatory reaction.* Am J Pathol, 2002. **160**(3): p. 1181-91.

5. Wu, H., et al., *TLR4 activation mediates kidney ischemia/reperfusion injury.* J Clin Invest, 2007. **117**(10): p. 2847-59.

6. Chamaillard, M., et al., *An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid.* Nat Immunol, 2003. **4**(7): p. 702-7.

7. Girardin, S.E., et al., *Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan.* Science, 2003. **300**(5625): p. 1584-7.

8. Girardin, S.E., et al., *Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection.* J Biol Chem, 2003. **278**(11): p. 8869-72.

9. Inohara, N., et al., *Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease.* J Biol Chem, 2003. **278**(8): p. 5509-12.

10. Stroo, I., et al., *Phenotyping of Nod1/2 double deficient mice and characterization of Nod1/2 in systemic inflammation and associated renal disease.* Biol Open, 2012. **1**(12): p. 1239-47.

11. Sabbah, A., et al., *Activation of innate immune antiviral responses by Nod2.* Nat Immunol, 2009. **10**(10): p. 1073-80.

12. Cheng, G., et al., *Activation of the nucleotide oligomerization domain signaling pathway by the non-bacterially derived xanthone drug 5'6-dimethylxanthenone-4-acetic acid (Vadimezan).* J Biol Chem, 2010. **285**(14): p. 10553-62.

13. Shigeoka, A.A., et al., *Nod1 and nod2 are expressed in human and murine renal tubular epithelial cells and participate in renal ischemia reperfusion injury.* J Immunol, 2010. **184**(5): p. 2297-304.

14. Dharancy, S., et al., *Neutrophil migration during liver injury is under nucleotide-binding oligomerization domain 1 control.* Gastroenterology, 2010. **138**(4): p. 1546-56, 1556.e1-5.

15. Inohara, N., et al., *Nod1, an Apaf-1-like activator of caspase-9 and nuclear factor-kappaB.* J Biol Chem, 1999. **274**(21): p. 14560-7.

16. Uehara, A., et al., *Various human epithelial cells express functional Toll-like receptors, NOD1 and NOD2 to produce anti-microbial peptides, but not proinflammatory cytokines.* Mol Immunol, 2007. **44**(12): p. 3100-11.

17. Anders, H.J. and D.A. Muruve, *The inflammasomes in kidney disease.* J Am Soc Nephrol, 2011. **22**(6): p. 1007-18.

18. Du, P., et al., *NOD2 promotes renal injury by exacerbating inflammation and podocyte insulin resistance in diabetic nephropathy.* Kidney Int, 2013. **84**(2): p. 265-76.

19. Pulskens, W.P., et al., *Nlrp3 prevents early renal interstitial edema and vascular permeability in unilateral ureteral obstruction.* PLoS One, 2014. **9**(1): p. e85775.

20. Pulskens, W.P., et al., *TLR4 promotes fibrosis but attenuates tubular damage in progressive renal injury.* J Am Soc Nephrol, 2010. **21**(8): p. 1299-308.

21. Vilaysane, A., et al., *The NLRP3 inflammasome promotes renal inflammation and contributes to CKD.* J Am Soc Nephrol, 2010. **21**(10): p. 1732-44.

22. Geddes, K., et al., *Nod1 and Nod2 regulation of inflammation in the Salmonella colitis model.* Infect Immun, 2010. **78**(12): p. 5107-15.

23. Humphreys, B.D., et al., *Chronic epithelial kidney injury molecule-1 expression causes murine kidney fibrosis.* J Clin Invest, 2013. **123**(9): p. 4023-35.

24. Skuginna, V., et al., *Toll-like receptor signaling and SIGIRR in renal fibrosis upon unilateral ureteral obstruction.* PLoS One, 2011. **6**(4): p. e19204.

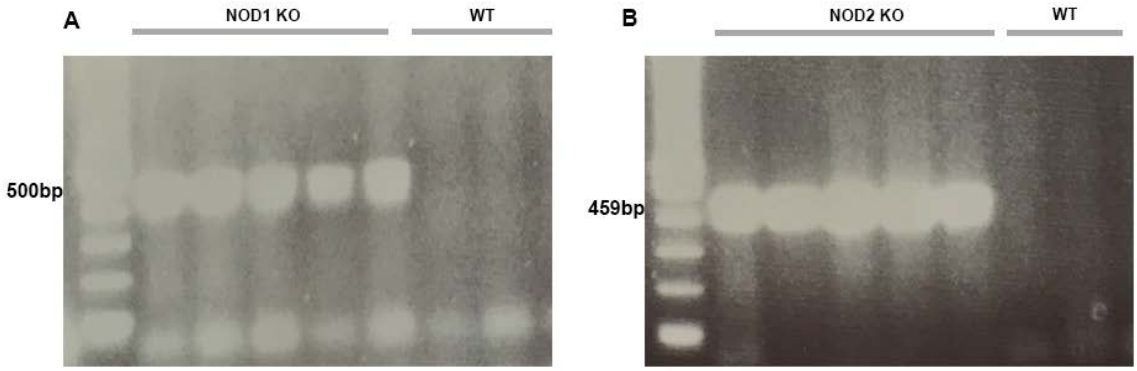
25. Campbell, M.T., et al., *Toll-like receptor 4: a novel signaling pathway during renal fibrogenesis.* J Surg Res, 2011. **168**(1): p. e61-9.

26. Leemans, J.C., et al., *Renal-associated TLR2 mediates ischemia/reperfusion injury in the kidney.* J Clin Invest, 2005. **115**(10): p. 2894-903.

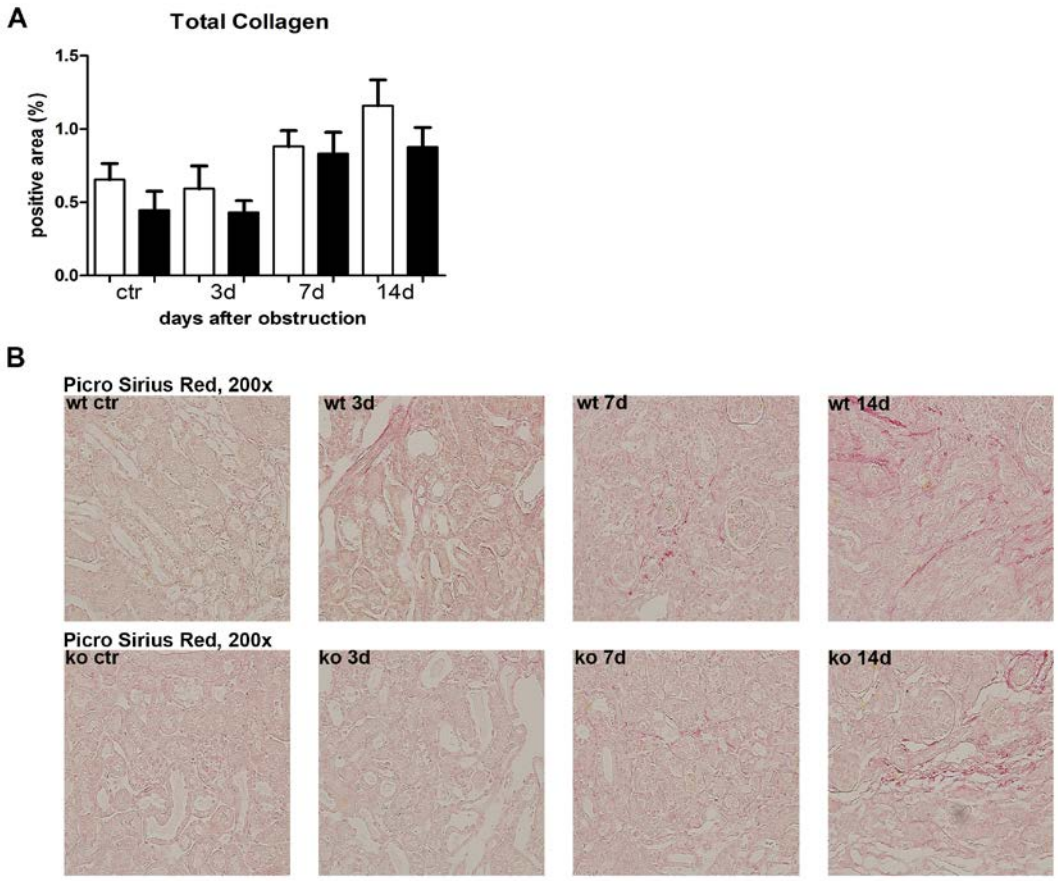
27. Kim, M., et al., *Isoflurane activates intestinal sphingosine kinase to protect against renal ischemia-reperfusion-induced liver and intestine injury.* Anesthesiology, 2011. **114**(2): p. 363-73.

28. Eddy, A.A., et al., *Investigating mechanisms of chronic kidney disease in mouse models.* Pediatr Nephrol, 2012. **27**(8): p. 1233-47.

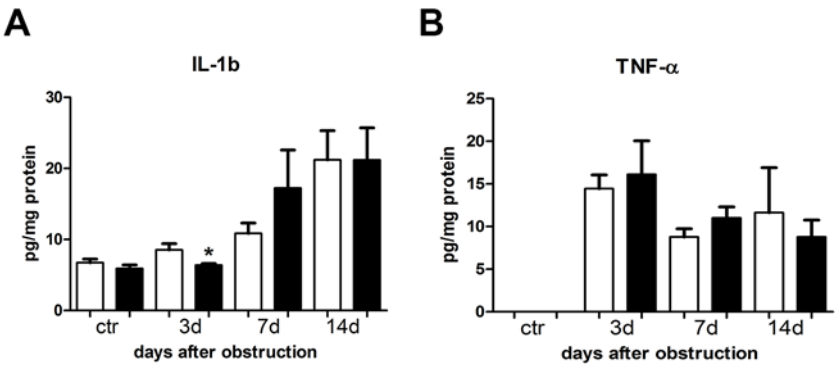
Supplementary information



Supplementary figure 1: **The genotype of NOD1/2 DKO mice.** Genomic DNA from mice was amplified by PCR with specific primers to detect the disrupted sequences on a 1% agarose gel with a 100bp marker. First 5 bands are the KO mice and the last bands are the WT mice in A and B. N=5/3 per group.



Supplementary figure 2: **Total collagen in kidneys of WT (white bars) and NOD1/2 DKO (black bars) mice after 0, 3, 7, and 14 days following obstruction.** Total collagen was assessed by Picro Sirius Red staining which was digitally analysed (A, B). Data are expressed as mean ± SEM. N=9/group.



Supplementary figure 3: **Renal inflammation in WT (white bars) and NOD1/2 DKO (black bars) mice after 0, 3, 7, and 14 days following obstruction.** IL-1b (A) and TNF-α (B) were measured in total kidney homogenates with specific ELISAs. Data are expressed as mean ± SEM. Results were analysed with the non-parametric two-tailed Mann-Whitney U-test. *$P<0.05$. N=9/group.

Chapter 6

General Discussion and Summary

General Discussion and Summary

In chapter 2, we aimed to investigate the potential effect of calcineurin inhibitor Tacrolimus on host immune response during UTI, given the high susceptibility of UTIs after renal transplantation[1-4]. We found that Tacrolimus impairs several crucial parts of the antimicrobial defense mechanism in mice. Tacrolimus pre-treated mice displayed higher bacterial burden (cystitis, pyelonephritis and bacteraemia) upon UTI. Granulocytes and macrophages are the key players against evading *E. coli* in the urinary tract[5-7]. The most populous immune cells in the naïve bladder are F4/80+ resident macrophages, involved in early recognition of *E. coli*, followed by release of TNF-α, MIP-2 and KC to recruit granulocytes from circulation into the infected urothelium[5]. In our study, we found that granulocytes from Tacrolimus group were less efficient in phagocytizing *E. coli*; they produced less MPO upon *E. coli* stimulation and the expression of CXCR2 chemokine receptor was reduced on circulating granulocytes. CXCR2 KO mice have been shown to have delayed granulocyte transmigration across the urothelium resulting in higher susceptibility to UTI[8, 9]. Initial immune response to *E. coli* in the bladder has been shown to be TLR5 dependent[10]. We found that TLR5 expression was significantly decreased on bladder macrophages *in vivo* during UTI. Moreover, Tacrolimus pre-treated macrophages were less responsive to LPS stimulation, a reflected by decreased release of pro-inflammatory mediators. Tacrolimus inhibits calcineurin, which is a negative regulator of TLR-pathway[11]. Therefore, we hypothesized that calcineurin inhibition by Tacrolimus would lead to activation of NF-κB and subsequently to upregulation of TLR negative regulators (endotoxin tolerance). Indeed, we found that IRAK-M, A20, ATF3 and SOCS1 were upregulated upon Tacrolimus stimulation in macrophages. Endotoxin tolerance is related to impaired resistance against several infections[12, 13]. Taken together, our data show that similarly to endotoxin tolerance-phenomena, Tacrolimus interferes with TLR-signalling, generating an immunotolerant state that can explain impaired granulocyte and macrophage functions.

In chapter 3, we reveal mechanistic insights into the gut-kidney axis during AKI. The impact of microbiome in shaping the immune system outside the gut has been intensively studies in the past decades. Several studies discovered that gut microbiome affect the immune response to different inflammatory disorders[14-17]. We show that gut microbiota depletion is protective against renal I\R-injury as reflected by reduced renal damage, dysfunction and inflammation.

We found decreased levels of renal inflammatory mediators early after ischemic damage in commensal-depleted mice. This was due to decreased expression of F4/80 and CX3CR1 on renal resident macrophages from commensal-depleted mice. Moreover, migration capacity and expression levels of F4/80, CX3CR1 and CCR2 was reduced in bone-marrow monocytes from commensal-depleted mice. Reintroduction of microbiota into antibiotic-treated mice abolished the protective effect of microbiota-depletion upon I\R injury. Our data highlights the importance of gut microbiota in priming maturation status of monocytes/macrophages. In absence of this priming stimulus, monocytes/macrophages are less active and therefore respond weaker to ischemic damage. Identifying commensal-derived components that are responsible for priming immune cells is essential, it can be used as a tool in order to shape immune reactions during inflammation. One study has shown that peptidoglycan can translocate from the gut to circulation in germ-free mice that were colonized with *E. coli* for 3 days[17]. We however, could not detect levels of peptidoglycan or LPS in the plasma of control mice, suggesting that other gut-derived products may be involved in priming of cells or that the levels are too low to be detected but sufficient for a tonic activation of monocytes/macrophages. In conclusion, we show that commensals are involved in priming macrophage functions to become fully mature and contribute to the initiation of inflammation and subsequent nephropathy after renal I/R-injury. These findings present first steps towards understanding how to tailor microbiota-related therapies in the future.

In chapter 4, we investigated the mechanism behind platelet activation during I/R-injury and how platelet activation contributes to tissue injury. Necrotic TECs induced by ischemic damage are highly immunostimulatory by releasing DAMPs such as heat shock proteins, HMGB1 proteins and DNA[18-20]. These DAMPs activate immune cells via PRRs in order to respond to tissue damage eventually leading to inflammation[20]. Recently it has become clear that platelets express PRRs as well and therefore are able to respond to DAMPs[21]. In our study we found that the release of extracellular DNA by necrotic TECs triggers activation of platelets, leading to platelet-granulocyte interaction and thereby stimulating neutrophil extracellular traps (NETs). NETs are composed of cytotoxic web of DNA strands, histones and granular proteins and have been linked to pathogenesis of inflammatory diseases such as sepsis and transfusion-related acute lung injury[22-24]. For the first time, our study demonstrates the presence of NETs in renal tissue following renal I/R-injury. Treatment with Clopidogrel significantly reduced NET formation in renal tissue after I/R-injury. Taken together, our

results indicate that platelet activation can be triggered by extracellular DNA derived from necrotic renal cells, leading to platelet-granulocyte interaction and generating NET formation, which could worsen renal injury during I/R.

In chapter 5, we investigated the role of NOD1 and NOD2 in chronic renal inflammation, injury and fibrosis by subjecting WT and NOD1/2 DKO mice at various time points to UUO model. PPRs including TLRs and NLRs are able to sense endogenous ligands released upon tissue damage, resulting in sterile inflammation and eventually irreversible renal disease[19]. NOD1 and NOD2 are known to contribute in pathogenesis of several inflammatory disease, including AKI[25]. The aim of this study was therefore to unravel the contribution of NOD1 and NOD2 in renal interstitial fibrosis and obstructive nephropathy. In our study we did not find a significant role of NOD1 and NOD2 in the development of tubulointerstitial fibrosis, inflammation nor in the progression of renal damage upon UUO-induced injury. Others have shown that TLR4 and NLRP3 have a central role in renal inflammation, fibrosis and tubular damage during obstructive nephropathy[26, 27]. One limitation of our study is the use of double knockout mice, therefore we cannot rule out the possibility that either NOD1 or NOD2 play a different role in UUO. Taken together, our data do not show a functional role for NOD1/2 in kidney injury and fibrosis following CKD induced by ureteral obstruction.

References

1. Chuang, P, Parikh, CR, Langone, A: Urinary tract infections after renal transplantation: a retrospective review at two US transplant centers. *Clinical transplantation*, 19**:** 230-235, 2005.
2. Fishman, JA, Rubin, RH: Infection in organ-transplant recipients. *The New England journal of medicine*, 338**:** 1741-1751, 1998.
3. Golebiewska, J, Debska-Slizien, A, Komarnicka, J, Samet, A, Rutkowski, B: Urinary tract infections in renal transplant recipients. *Transplantation proceedings*, 43**:** 2985-2990, 2011.
4. Rubin, RH: Infectious disease complications of renal transplantation. *Kidney international*, 44**:** 221-236, 1993.
5. Mora-Bau, G, Platt, AM, van Rooijen, N, Randolph, GJ, Albert, ML, Ingersoll, MA: Macrophages Subvert Adaptive Immunity to Urinary Tract Infection. *PLoS pathogens*, 11**:** e1005044, 2015.
6. Schiwon, M, Weisheit, C, Franken, L, Gutweiler, S, Dixit, A, Meyer-Schwesinger, C, Pohl, JM, Maurice, NJ, Thiebes, S, Lorenz, K, Quast, T, Fuhrmann, M, Baumgarten, G, Lohse, MJ, Opdenakker, G, Bernhagen, J, Bucala, R, Panzer, U, Kolanus, W, Grone, HJ, Garbi, N, Kastenmuller, W, Knolle, PA, Kurts, C, Engel, DR: Crosstalk between sentinel and helper macrophages permits neutrophil migration into infected uroepithelium. *Cell*, 156**:** 456-468, 2014.
7. Spencer, JD, Schwaderer, AL, Becknell, B, Watson, J, Hains, DS: The innate immune response during urinary tract infection and pyelonephritis. *Pediatric nephrology (Berlin, Germany)*, 29**:** 1139-1149, 2014.
8. Frendeus, B, Godaly, G, Hang, L, Karpman, D, Lundstedt, AC, Svanborg, C: Interleukin 8 receptor deficiency confers susceptibility to acute experimental pyelonephritis and may have a human counterpart. *The Journal of experimental medicine*, 192**:** 881-890, 2000.
9. Olszyna, DP, Florquin, S, Sewnath, M, Branger, J, Speelman, P, van Deventer, SJ, Strieter, RM, van der Poll, T: CXC chemokine receptor 2 contributes to host defense in murine urinary tract infection. *The Journal of infectious diseases*, 184**:** 301-307, 2001.
10. Andersen-Nissen, E, Hawn, TR, Smith, KD, Nachman, A, Lampano, AE, Uematsu, S, Akira, S, Aderem, A: Cutting edge: Tlr5-/- mice are more susceptible to Escherichia coli urinary tract infection. *Journal of immunology (Baltimore, Md : 1950)*, 178**:** 4717-4720, 2007.
11. Kang, YJ, Kusler, B, Otsuka, M, Hughes, M, Suzuki, N, Suzuki, S, Yeh, WC, Akira, S, Han, J, Jones, PP: Calcineurin negatively regulates TLR-mediated activation pathways. *Journal of immunology (Baltimore, Md : 1950)*, 179**:** 4598-4607, 2007.
12. Youngner, JS, Stinebring, WR: Interferon appearance stimulated by endotoxin, bacteria, or viruses in mice pre-treated with Escherichia coli endotoxin or infected with Mycobacterium tuberculosis. *Nature*, 208**:** 456-458, 1965.
13. Mason, CM, Dobard, E, Summer, WR, Nelson, S: Intraportal lipopolysaccharide suppresses pulmonary antibacterial defense mechanisms. *The Journal of infectious diseases*, 176**:** 1293-1302, 1997.
14. Abt, MC, Osborne, LC, Monticelli, LA, Doering, TA, Alenghat, T, Sonnenberg, GF, Paley, MA, Antenus, M, Williams, KL, Erikson, J, Wherry, EJ, Artis, D: Commensal bacteria calibrate the activation threshold of innate antiviral immunity. *Immunity*, 37**:** 158-170, 2012.
15. Benson, A, Pifer, R, Behrendt, CL, Hooper, LV, Yarovinsky, F: Gut commensal bacteria direct a protective immune response against Toxoplasma gondii. *Cell host & microbe*, 6**:** 187-196, 2009.

16. Clarke, TB: Early innate immunity to bacterial infection in the lung is regulated systemically by the commensal microbiota via nod-like receptor ligands. *Infection and immunity,* 82**:** 4596-4606, 2014.
17. Clarke, TB, Davis, KM, Lysenko, ES, Zhou, AY, Yu, Y, Weiser, JN: Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity. *Nature medicine,* 16**:** 228-231, 2010.
18. Allam, R, Scherbaum, CR, Darisipudi, MN, Mulay, SR, Hagele, H, Lichtnekert, J, Hagemann, JH, Rupanagudi, KV, Ryu, M, Schwarzenberger, C, Hohenstein, B, Hugo, C, Uhl, B, Reichel, CA, Krombach, F, Monestier, M, Liapis, H, Moreth, K, Schaefer, L, Anders, HJ: Histones from dying renal cells aggravate kidney injury via TLR2 and TLR4. *Journal of the American Society of Nephrology : JASN,* 23**:** 1375-1388, 2012.
19. Leemans, JC, Kors, L, Anders, HJ, Florquin, S: Pattern recognition receptors and the inflammasome in kidney disease. *Nature reviews Nephrology,* 10**:** 398-414, 2014.
20. Thurman, JM: Triggers of inflammation after renal ischemia/reperfusion. *Clinical immunology (Orlando, Fla),* 123**:** 7-13, 2007.
21. Andonegui, G, Kerfoot, SM, McNagny, K, Ebbert, KV, Patel, KD, Kubes, P: Platelets express functional Toll-like receptor-4. *Blood,* 106**:** 2417-2423, 2005.
22. Caudrillier, A, Kessenbrock, K, Gilliss, BM, Nguyen, JX, Marques, MB, Monestier, M, Toy, P, Werb, Z, Looney, MR: Platelets induce neutrophil extracellular traps in transfusion-related acute lung injury. *The Journal of clinical investigation,* 122**:** 2661-2671, 2012.
23. Clark, SR, Ma, AC, Tavener, SA, McDonald, B, Goodarzi, Z, Kelly, MM, Patel, KD, Chakrabarti, S, McAvoy, E, Sinclair, GD, Keys, EM, Allen-Vercoe, E, Devinney, R, Doig, CJ, Green, FH, Kubes, P: Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. *Nature medicine,* 13**:** 463-469, 2007.
24. McDonald, B, Urrutia, R, Yipp, BG, Jenne, CN, Kubes, P: Intravascular neutrophil extracellular traps capture bacteria from the bloodstream during sepsis. *Cell host & microbe,* 12**:** 324-333, 2012.
25. Shigeoka, AA, Kambo, A, Mathison, JC, King, AJ, Hall, WF, da Silva Correia, J, Ulevitch, RJ, McKay, DB: Nod1 and nod2 are expressed in human and murine renal tubular epithelial cells and participate in renal ischemia reperfusion injury. *Journal of immunology (Baltimore, Md : 1950),* 184**:** 2297-2304, 2010.
26. Pulskens, WP, Rampanelli, E, Teske, GJ, Butter, LM, Claessen, N, Luirink, IK, van der Poll, T, Florquin, S, Leemans, JC: TLR4 promotes fibrosis but attenuates tubular damage in progressive renal injury. *Journal of the American Society of Nephrology : JASN,* 21**:** 1299-1308, 2010.
27. Campbell, MT, Hile, KL, Zhang, H, Asanuma, H, Vanderbrink, BA, Rink, RR, Meldrum, KK: Toll-like receptor 4: a novel signaling pathway during renal fibrogenesis. *The Journal of surgical research,* 168**:** e61-69, 2011.

Chapter 7

Nederlandse samenvatting

PhD portfolio

List of publications

Curriculum Vitae

Acknowledgments

Nederlandse samenvatting

Basisprincipes van nier anatomie en fysiologie

De nieren zijn twee boonvormige organen, aan weerszijden van de ruggengraat gelegen in de retroperitoneale ruimte. Zij zijn ongeveer 11 cm lang, 6 cm breed en 3-5 cm dik en wegen 115-175 gram. De primaire functie van dit complexe orgaan is om de homeostase van het lichaam te behouden via een zeer gespecialiseerde microscopische anatomie. De kleinste functionele eenheid van de nier wordt een nefron genoemd. Een gezonde volwassen menselijke nier bestaat uit ongeveer 1 miljoen nefronen. Elk nefron omvat een glomerulus en een buisvormig compartiment (proximale tubulus, lis van Henle, distale tubulus en verzamelbuis). Circulerend bloed wordt gefilterd door de glomerulus die wordt omringd door de kapsel van Bowman die de verbinding vormt met de proximale tubulus. Het resulterende filtraat, vrij van cellen en grote eiwitten, stroomt door de verschillende delen van de tubulus waar reabsorptie van water, zouten en andere essentiële moleculen plaatsvindt, terwijl afvalproducten en toxines worden afgescheiden in de verzamelbuis. Hierna komt de urine via de urineleider in de blaas. Filtratie, reabsorptie en secretie door de nieren, houden het lichaam in evenwicht met betrekking tot water, mineralen, elektrolyten en waterstofionenconcentratie. Afgezien daarvan dienen de nieren als een endocrien orgaan door erytropoëtine, renine, prostaglandinen en vitamine D3 aan te maken.

Nierziekten die relevant zijn voor dit proefschrift

Urineweginfectie

De therapie bij uitstek voor patiënten met terminale nierziekte (ESRD) is een niertransplantatie. Succesvolle niertransplantatie is afhankelijk van levenslang gebruik van adequate immunosuppressie om transplantaatafstoting te voorkomen. Immuungecompromitteerde patiënten zoals niertransplantatiepatiënten zijn kwetsbaar voor infectieuze complicaties na transplantatie. Urineweginfecties (UTI's) zijn de meest voorkomende bacteriële infecties met een prevalentie van 60% gedurende het eerste jaar na niertransplantaties. De meest relevante pathogenen zijn *Escherichia coli* (*E. coli*), *Enterococcus* en *Staphylococcus*. Vrouwelijk geslacht, gevorderde leeftijd, , episodes van acute afstoting en de mate van immunosuppressie zijn risicofactoren voor UTI bij ontvangers van niertransplantaten. UTI's kunnen vaak leiden tot acute graft pyelonefritis, wat wordt

beschouwd als een potentiële risicofactor voor slechtere transplantaatfunctie. In de Eerste maand na de transplantatie is de met bacteriële infectie geassocieerde mortaliteit 11%. UTI's zijn de meest voorkomende bron van sepsis bij getransplanteerden. Het standaard gebruik van trimethoprim-sulfamethoxazol-profylaxe (TMP-SMZ) in de eerste zes maanden na niertransplantatie lijkt niet effectief te zijn om de hoge incidentie van UTI's te voorkomen. Gebruik van routinematige TMP-SMZ profylaxe bij niertransplantatiepatiënten is geassocieerd met een toenemende hoeveelheid resistente *E. coli*-isolaten tegen TMP-SMX. UTI kan worden bestudeerd in een experimenteel muismodel waarbij infectie wordt geïnitieerd door transurethrale inoculatie van *E. coli*-suspensie in de blaas. Bacteriegroei en ontstekingsreacties kunnen 24 en 48 uur later worden bepaald. Dit model is gebruikt in hoofdstuk 2 van dit proefschrift, waarin we de mogelijke effecten van Tacrolimus op de immuunrespons van de gastheer hebben onderzocht.

Acute nierschade

Acute nierschade (AKI), een plotselinge daling van de nierfunctie, gekenmerkt door verhoogde serumspiegels van creatinine en ureum, is een veel voorkomende klinische complicatie die 5% van de gehospitaliseerde patiënten treft. AKI-gerelateerde mortaliteit is 50-80% bij ernstig zieke patiënten. Ischemie reperfusie (I/R)-schade is de overheersende oorzaak van AKI, die optreedt tijdens shock en transplantatie. I/R-schade wordt gedefinieerd als een abrupte en voorbijgaande daling van de bloedtoevoer, waardoor de nieren een tekort aan voedingsstoffen en zuurstof ondervinden, gevolgd door herstel van de bloedstroom, de reperfusiefase. Ischemie induceert celdood van renale tubulaire epitheliale cellen (TECs), hetzij via apoptose of necrose, resulterend in lokale afgifte van schade-geassocieerde moleculaire patronen (DAMPs) zoals HMGB1, heat shock-eiwitten en mitochondriaal DNA. TEC's worden echter niet alleen passief beschadigd door letsel, maar nemen ook actief deel aan de ontstekingsreactie. DAMPs activeren TEC's en immuun cellen via PRR's, waardoor een ontstekingstoestand ontstaat die het kenmerk is van AKI. De vroege immuunrespons tijdens I/R-schade wordt geïnitieerd door TEC's en macrofagen in de nier, die pro-inflammatoire mediatoren zoals TNF-a, MCP-1 en IL-8 afgeven. Vervolgens wordt één dag na I/R-schade het ontstekingsmilieu voornamelijk gekenmerkt door een enorme influx van granulocyten, terwijl in een latere fase macrofagen en lymfocyten prominenter worden. Deze staat van steriele ontsteking na I/R-schade kan beschouwd worden als een tweesnijdend zwaard. Enerzijds is de ontstekingsreactie van cruciaal belang bij het herstellen van weefsel

na beschadiging. Anderzijds kan Langdurige en overmatige ontsteking juist meer schade veroorzaken en uiteindelijk leiden tot nierfibrose en terminaal nierfalen (ESDR). I/R-schade kan worden bestudeerd in een experimenteel diermodel waarbij unilaterale of bilaterale nierslagaders worden dichtgeklemd met kleine metalen klemmetjes gedurende een specifieke tijdsperiode. Dit model is gebruikt in hoofdstuk 3 en 4 van dit proefschrift, waarin we de impact van darmmicrobiota en bloedplaatjes tijdens I/R-schade respectievelijk bestudeerden.

Chronische nierziekte

Chronisch nierfalen (CKD) kent een wereldwijde prevalentie van 12% en vormt een belangrijke belasting voor de volksgezondheid, gekenmerkt door verminderde kwaliteit van leven, hoge morbiditeit en mortaliteit. CKD is een algemene term voor verschillende nierziekten met variabele etiologie. Criteria die CKD definiëren omvatten albuminurie en een glomerulaire filtratiesnelheid (GFR) van <60 ml/min/1,73m2 gedurende een periode langer dan 3 maanden. Verlies van nefronen als gevolg van hoge leeftijd, acuut of chronisch nierschade veroorzaakt door toxines of ziekten zoals UTI, AKI, diabetes mellitus type 2, obesitas en hypertensie zijn risicofactoren voor de ontwikkeling en progressie van CKD. Nierfibrose is de ”final common pathway” voor alle soorten CKD, leidend tot ESRD, een onomkeerbare aandoening waarvoor niervervangende therapie vereist is. Unilaterale ureterobstructie (UUO) bij muizen is een vaak gebruikt model om mechanismen van nierfibrose te onderzoeken. Het UUO-model bootst chronische obstructieve nefropathie na, wat een belangrijke oorzaak is van nierinsufficiëntie bij kinderen. Het UUO-model induceert verschillende kenmerken van nierfibrose, waaronder tubulo-interstitiële ontsteking, accumulatie van myofibroblasten, verhoogde afzetting van extracellulaire matrix (ECM) en tubulaire atrofie. De meeste infiltrerende leukocyten in dit model zijn macrofagen die een dubbele rol spelen door ofwel fibrose te bevorderen of weefselschade in een later stadium op te lossen. Macrofagen bevorderen voornamelijk fibrose door de afgifte van transforming growth factor-β (TGFβ), dat -afzetting van ECM genereert door de eiwitsynthese te verhogen en de afbraak van matrixeiwitten te verminderen. In een later stadium van de verwonding zijn macrofagen betrokken bij fagocytose van ECM-fragmenten en bevorderen hierbij weefselherstel. Macrofagen zijn ook betrokken bij het bevorderen van TEC-apoptose die samen met een afgenomen TEC-proliferatie de balans naar celdood verschuift, wat

uiteindelijk resulteert in tubulo-interstitiële atrofie. In Hoofdstuk 5 van dit proefschrift hebben we het UUO-model gebruikt om de rol van NOD1/2 in nierschade en fibrose te ontrafelen.

In hoofdstuk 2, hebben we het mogelijke effect onderzocht van calcineurineremmer Tacrolimus op de immuunreactie van de gastheer tijdens UTI, gezien de hoge vatbaarheid voor UTI's na niertransplantatie. We hebben ontdekt dat Tacrolimus verschillende cruciale onderdelen van het antimicrobiële afweermechanisme bij muizen schaadt. Vooraf met Tacrolimus behandelde muizen vertoonden een hogere bacteriële belasting (cystitis, pyelonefritis en bacteriëmie) na UTI dan niet-behandelde muizen. Granulocyten en macrofagen zijn de belangrijkste spelers in de afweer tegen *E. coli* in de urinewegen. De meest voorkomende immuun cellen in de naïeve blaas zijn F4/80+ residente macrofagen, die betrokken zijn bij vroege herkenning van *E. coli*, gevolgd door afgifte van TNF-α, MIP-2 en KC om granulocyten uit circulatie in het geïnfecteerde urotheel te rekruteren. In onze studie vonden we dat granulocyten uit de Tacrolimus-groep minder efficiënt waren in het fagocyteren van *E. coli*; ze produceerden minder MPO na stimulering met *E. coli* en de expressie van CXCR2 chemokine receptor was verminderd op circulerende granulocyten. Van CXCR2 KO-muizen is aangetoond dat de transmigratie van granulocyten door het urotheel vertraagd is, resulterend in een hogere gevoeligheid voor UTI. Er is aangetoond dat de initiële immuunrespons op *E. coli* in de blaas TLR5-afhankelijk is. We vonden dat TLR5-expressie tijdens UTI *in vivo* significant verminderde op blaasmacrofagen. Bovendien waren met Tacrolimus voorbehandelde macrofagen minder gevoelig voor LPS-stimulatie, hetgeen werd weerspiegeld door een verminderde afgifte van pro-inflammatoire mediatoren. Tacrolimus remt calcineurine, wat een negatieve regulator is van de TLR-pathway. Daarom veronderstelden we dat calcineurine-inhibitie door Tacrolimus zou leiden tot activatie van NF-KB en vervolgens tot verhoogde expressie van TLR-negatieve regulatoren (endotoxinetolerantie). Inderdaad, we ontdekten dat IRAK-M, A20, ATF3 en SOCS1 verhoogd werden door Tacrolimus-stimulatie in macrofagen. Endotoxinetolerantie is gerelateerd aan verminderde resistentie tegen verschillende infecties. Samengevat laten onze gegevens zien dat Tacrolimus, vergelijkbaar met endotoxinetolerantie, interfereert met TLR-signalering, en een immunotolerante toestand genereert die veranderde granulocyten- en macrofaagfuncties kan verklaren.

In hoofdstuk 3, onthullen we mechanistische inzichten in de darm-nier-as tijdens AKI. De impact van het microbioom (darm bacteriën) op het immuunsysteem buiten het darmkanaal is

intensief onderzocht in de afgelopen decennia. Verschillende studies hebben ontdekt dat het darmmicrobioom de immuunrespons op verschillende ontstekingsaandoeningen beïnvloedt. We laten zien dat de afwezigheid van de darmflora beschermend is tegen I/R-schade aan de nieren, zoals wordt weerspiegeld door verminderde nierbeschadiging, betere nierfunctie en minder ontsteking. We vonden verlaagde niveaus van renale inflammatoire mediatoren vroeg na ischemische schade in muizen zonder darmmicrobioom. Dit was het gevolg van verlaagde expressie van F4/80 en CX3CR1 op macrofagen in de nier van muizen zonder darm bacteriën. Bovendien waren de migratiecapaciteit en expressieniveaus van F4/80, CX3CR1 en CCR2 verlaagd in beenmergmonocyten van muizen zonder darmmicrobioom. Herintroductie van microbiota in met antibiotica behandelde muizen hief het beschermende effect van microbiota afwezigheid bij I/R-schade op. Onze resultaten benadrukken het belang van darmmicrobiotica in de priming-rijpingstoestand van monocyten/macrofagen. Bij afwezigheid van deze primingstimulus zijn monocyten/macrofagen minder actief en reageren daarom zwakker op ischemische schade. Het identificeren van de darmbacteriecomponenten die verantwoordelijk zijn voor het primen van immuun cellen is essentieel, omdat het kan worden gebruikt als een hulpmiddel om immuunreacties tijdens ontstekingen vorm te geven. Eén onderzoek heeft aangetoond dat peptidoglycaan zich van het maagdarmkanaal naar de bloedsomloop kan verplaatsen in kiemvrije muizen die 3 dagen lang gekoloniseerd waren met *E. coli*. Wij konden echter geen peptidoglycan of LPS in het plasma van controle muizen detecteren, wat suggereert dat ook andere van de darm flora afgeleide producten betrokken kunnen zijn bij het primen van cellen of dat de levels te laag zijn om te worden gedetecteerd maar voldoende voor activatie van monocyten/macrofagen. Concluderend laten we zien dat darm flora betrokken zijn bij het primen van macrofaag functies om volledig te rijpen en bij te dragen aan het starten van ontsteking en daaropvolgende nefropathie na renale I/R-schade. Deze bevindingen vormen de eerste stap naar een beter begrip van hoe microbiota-gerelateerde therapieën in de toekomst kunnen worden aangepast.

In hoofdstuk 4, onderzochten we het mechanisme achter bloedplaatjesactivatie tijdens I/R-schade en hoe activering van bloedplaatjes bijdraagt aan weefselbeschadiging. Necrotische TEC's geïnduceerd door ischemische schade zijn zeer immunostimulerend door DAMP's vrij te maken, zoals heat shock-eiwitten, HMGB1-eiwitten en DNA. Deze DAMPs activeren immuun cellen via PRR's om te reageren op weefselbeschadiging die uiteindelijk leidt tot ontsteking. Onlangs is duidelijk geworden dat bloedplaatjes PRR's ook tot expressie brengen

en daarom in staat zijn te reageren op DAMPs. In onze studie vonden we dat de afgifte van extracellulair DNA door necrotische TEC's activering van bloedplaatjes triggert, leidend tot interactie van bloedplaatjes en granulocyten met vorming van neutrophil extracellular traps (NET's) tot gevolg. NET's zijn samengesteld uit een cytotoxisch netwerk van DNA-strengen, histonen en granulaire eiwitten en zijn aanvankelijk in verband gebracht met de pathogenese van ontstekingsziekten zoals sepsis en aan transfusie gerelateerd acuut longletsel. Ons onderzoek is het eerste dat aanwezigheid van NET's in nierweefsel na renale I/R-schade aantoont. Behandeling met Clopidogrel verminderde de NET-vorming significant in nierweefsel na I/R-schade. Alles bij elkaar, geven onze resultaten aan dat de activering van bloedplaatjes kan worden veroorzaakt door extracellulair DNA dat is afgeleid van necrotische niercellen, wat leidt tot de interactie van bloedplaatjes en granulocyten met NET-vorming als gevolg, wat nierschade tijdens I/R kan verergeren.

In hoofdstuk 5, onderzochten we de rol van NOD1 en NOD2 bij chronische nierontsteking, nierschade en fibrose door WT en NOD1/2 DKO-muizen op verschillende tijdstippen aan het UUO-model te onderwerpen. PPR's, waaronder TLR's en NLR's, kunnen endogene liganden die vrijkomen bij weefselschade detecteren, resulterend in steriele ontsteking en uiteindelijk onomkeerbare nierziekte. Van NOD1 en NOD2 is bekend dat ze bijdragen aan de pathogenese van verschillende inflammatoire ziekten, waaronder AKI. Het doel van deze studie was daarom om de bijdrage van NOD1 en NOD2 aan renale interstitiële fibrose en obstructieve nefropathie te ontrafelen. In onze studie vonden we geen significante rol van NOD1 en NOD2 in de ontwikkeling van tubulo-interstitiële fibrose en ontsteking, noch in de progressie van nierschade na door UUO geïnduceerd letsel. Anderen hebben aangetoond dat TLR4 en NLRP3 een centrale rol spelen bij nierontsteking, fibrose en tubulaire schade tijdens obstructieve nefropathie. Een beperking van onze studie is het gebruik van dubbele knock-out muizen, daarom kunnen we niet uitsluiten dat NOD1 of NOD2 een andere rol spelen in UUO. Samengevat tonen onze gegevens geen functionele rol voor NOD1/2 bij nierbeschadiging en fibrose na CKD geïnduceerd door ureterobstructie.

PhD Portfolio

Name: Diba Emal

PhD period: November 2012 – August 2017

PhD supervisors: Prof. Dr. S. Florquin
Dr. M. C. Dessing
Dr. J. C. Leemans

Post graduate courses	**Year**	**ECTS**
Winterschool nierstichting	2013	1
The AMC World of Science	2013	0.7
Laboratory animal science (art. 9)	2013	3
Advanced Immunology	2015	3
Infectious Diseases	2013	1
Clinical Data Management	2016	1

Seminars and masterclasses	**Year**	**ECTS**
Weekly Seminars in Pathology, AMC Amsterdam	2012-2017	7
Weekly Journal Clubs, AMC Amsterdam	2012-2017	8
AMC Infection Meeting (AIM), AMC Amsterdam	2016	1
De Anatomische Les, Masterclass of Prof. Martin Blaser, het Concertgebouw, Amsterdam	2014	0.5
Masterclass Prof. Roy Bloom, AMC Amsterdam	2016	0.5

Conferences	**Year**	**ECTS**
Nederlandse nefrologiedagen, Veldhoven, Nederland	2013 - 2017	3
ERA-EDTA Congress 2014, Amsterdam, Nederland	2014	1
Immunology 2015, New Orleans, USA	2015	1
TOLL 2015, Marbella Spain	2015	1
NFN Najaarssymposium, Utrecht Nederland	2013	0.5
NFN Benelux meeting, Eindhoven Nederland	2015	0.5
International Congress of Immunology 2016, Melbourne Australia	2016	1
Amsterdam Infection & Immunity meeting	2017	0.5

Oral presentations	**Year**	**ECTS**
Nefrologiedagen, Veldhoven, Nederland	2015	0.5
Nefrologiedagen, Veldhoven, Nederland (2 presentations)	2016	1
NFN Benelux meeting, Eindhoven, Nederland	2015	0.5
Seminars in Pathology, AMC Amsterdam	2016	0.5
AMC Infection Meeting (AIM), AMC Amsterdam	2016	0.5
Immunology 2015, New Orleans, USA	2015	0.5
International Congress of Immunology 2016, Melbourne Australia (2 presentations)	2016	1

Poster presentations	**Year**	**ECTS**
Immunology 2015, New Orleans, USA	2015	0.5
International Congress of Immunology 2016, Melbourne	2016	0.5

Awards	**Year**	**ECTS**
Abstract Award, Immunology 2015, New Orleans, USA	2015	
NFN Publicatieprijs 2017, Veldhoven, Nederland	2017	

Teaching/supervision	**Year**	**ECTS**
Internship Master student Bio-medical science	2016	
Thesis, Bachelor medicine student	2016	

List of Publications

1. **Emal, D,** Rampanelli, E, Claessen, N, Bemelman, FJ, Leemans, JC, Florquin, S, Dessing, MC: Calcineurin inhibitor Tacrolimus impairs host immune response against urinary tract infection. Scientific reports, 9: 106, 2019.

2. Stroo, I, **Emal, D**, Butter, LM, Teske, GJ, Claessen, N, Dessing, MC, Girardin, SE, Florquin, S, Leemans, JC: No difference in renal injury and fibrosis between wild-type and NOD1/NOD2 double knockout mice with chronic kidney disease induced by ureteral obstruction. BMC nephrology, 19: 78, 2018.

3. Tammaro, A, Kers, J, **Emal, D**, Stroo, I, Teske, GJD, Butter, LM, Claessen, N, Damman, J, Derive, M, Navis, GJ, Florquin, S, Leemans, JC, Dessing, MC: Effect of TREM-1 blockade and single nucleotide variants in experimental renal injury and kidney transplantation. Scientific reports, 6: 38275, 2016.

4. **Emal, D**, Rampanelli, E, Stroo, I, Butter, LM, Teske, GJ, Claessen, N, Stokman, G, Florquin, S, Leemans, JC, Dessing, MC: Depletion of Gut Microbiota Protects against Renal Ischemia-Reperfusion Injury. Journal of the American Society of Nephrology : JASN, 28: 1450-1461, 2017.

5. Jansen, MP, **Emal, D**, Teske, GJ, Dessing, MC, Florquin, S, Roelofs, JJ: Release of extracellular DNA influences renal ischemia reperfusion injury by platelet activation and formation of neutrophil extracellular traps. Kidney international, 91: 352-364, 2017.

6. Kolanowski, ST, Lissenberg-Thunnissen, SN, **Emal, D**, van Ham, SM, Ten Brinke, A: Monophosphoryl lipid A-induced pro-inflammatory cytokine expression does not require CD14 in primary human dendritic cells. Inflammation research : official journal of the European Histamine Research Society [et al], 65: 449-458, 2016.

Curriculum Vitae

Diba Emal was born on August 30, 1987 in Djawzdjan, Afghanistan. In 1998 she moved with her parents and siblings to the Netherlands. She obtained her VWO diploma at the Helen Parkhurst College in Almere, the Netherlands. Bachelor degree in Bio-medical science (Faculty of Earth and Life Sciences) was received in 2010 at the Free University of Amsterdam. Her Master's Degree in Oncology (Faculty of Medicine) was obtained in 2012 at the Free University of Amsterdam. During this study she performed a scientific research in the lab of Prof. Dr. T.D. de Gruijl at the department of Medical Oncology, CCA, VU Medical Center, Free University of Amsterdam. Her second scientific research was performed in the lab of Dr. A. ten Brinke at the department of Immunopathology, Sanquin Blood Supply, AMC, University of Amsterdam. Under supervision of Prof. Dr. M. van Egmond, she completed her master thesis on the subject of cancer immunotherapy at the department of Molecular Cell Biology and Immunology at the Free University of Amsterdam. In November 2012, she started her PhD project under supervision of Prof. Dr. S. Florquin, Dr. M.C. Dessing and Dr. J.C. Leemans at the deparment of Pathology, AMC, University of Amsterdam. The results of her PhD research project are described in this thesis. In January 2018, she made the transition from academia to the pharmaceutical industry, currently working in the Drug Regulatory Affairs at Sandoz B.V.

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