Immune-mediated mechanisms of (mal)adaptive renal tissue repair

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English summary

The incidence of renal diseases has increased significantly and it is a chief cause of morbidity and mortality worldwide. Specific treatment for patients with kidney diseases remains unavailable and current therapies, which are only supportive, consist of dialysis and renal transplantation. A better understanding of the pathogenesis of kidney diseases is required to develop new treatment opportunities. The innate immune system represents the first line of defense against invading pathogens but also plays a crucial role in renal tissue damage by alarming the host. Danger proteins released by damaged or inflammatory cells, the so-called Damage Associated Molecular Patterns (DAMPs), act as a ligand for Pattern Recognition Receptors (PRRs) and initiate an inflammatory reaction. This results in a clearance of invading pathogens that segues into tissue repair. Inflammatory cells are the main players in most inflammatory processes.

The danger protein S100A8/A9 and the innate immune receptor TREM-1 are expressed by neutrophils and monocyte/macrophages; both are able to elicit or amplify inflammatory signals through other PRRs. This thesis focuses on the role of the danger protein S100A8/A9 and the activating immune receptor TREM-1 in preclinical models of acute kidney injury (AKI) and chronic kidney disease (CKD).

In **Chapter 1** we give a brief overview of renal homeostasis and disease, together with a detailed description of the factors involved in the innate immune response, which was the main focus for the research described in this thesis.

Chapter 2 describes the contribution of S100A8/A9 in renal Ischemia/Reperfusion (IR)-induced AKI and it explores the significant role that danger proteins play in renal repair post-AKI. We subjected both wild type and S100A9 KO animals to bilateral clamping of the renal artery, followed by reperfusion for several days, in order to study the different phases of renal injury. We observed that S100A8/A9 expression was increased in the kidney during IR injury, especially 24 hours post-ischemia. This can most-likely be attributed to the infiltration of granulocytes that express high levels of S100A8/A9. Despite the increased expression of the protein 1 day post-IR, S100A9 KO animals displayed no major changes in renal inflammation or damage marker expression at this time point. However, we found that S100A8/A9 is crucial in limiting maladaptive repair post-AKI. Indeed, during the repair phase, S100A9 KO mice displayed signs of renal dysfunction and increased expression of tubular damage markers, which was also associated with an increased production of renal chemokines and cytokines. Finally, we show that S100A9 KO animals developed renal fibrosis, as shown by increased collagen deposition and TGFβ production. We found that the maladaptive repair phenotype observed in the S100A9 KO

animals, could be ascribed to an altered macrophage function. This is highly conceivable, considering the role that macrophages play in tissue repair has been well established. S100A9 KO macrophages revealed an aberrant M2-like phenotype *in vivo*, as shown by increased transcription of alternative M2 macrophage activation markers, namely Arg1, MGL1 and IRF5. Through *in vitro* experiments, we confirmed that upon M2 skewing with IL4 and IL13 cytokines, S100A9 KO macrophages displayed a phenotype similar the one observed *in vivo*. Possibly, during the repair phase, excessive M2 polarization, which is often associated with the release of pro-fibrotic factors, may further fuel the improper wound healing response, thereby leading to scar formation. Thus, S100A8/A9 is crucially involved in renal adaptive repair after ischemia, by controlling excessive M2 macrophage polarization and preventing renal fibrosis.

Macrophages are known to accumulate in the kidney during renal fibrosis and may also play a role in tissue degeneration. As we observed that S100A9 KO animals display increased fibrosis upon AKI due to excessive M2 macrophage polarization, we further investigated the role of S100A8/A9 in the development of renal fibrosis in **Chapter 3**. In this chapter we used the unilateral ureteral obstruction (UUO) in WT and S100A9 KO animals as a chronic model of renal damage, which leads to the development of severe tubulointerstital fibrosis. During the course of UUO, intrarenal S100A8/A9 positive cells increased overtime and co-expressed the granulocyte activation marker Ly6G. However, the infiltration of these inflammatory cells did not differ between WT and S100A9 KO animals . The expression profile of S100A8/A9 in the preclinical model was analogous to the one observed in patients with obstructive hydronephrosis. In contrast with previous evidences, S100A9 KO animals were protected against renal fibrosis, as displayed by decreased infiltration of myofibroblasts and collagen deposition. We found that the reduction in fibrosis, observed in KO animals that had sustained chronic damage, was related to the preservation of tubular epithelial cells (TEC) integrity. S100A9 KO animals displayed decreased tubular apoptosis and activation of critical epithelial-mesenchymal transition steps. In line with these findings, we observed that stimulation of TECs with recombinant S100A8/A9 induced cell cycle arrest and signs of dedifferentiation, including decreased expression of cell junction and adhesion molecules that are essential in supporting tubular structure and function. When exposed to additional stimulation with the profibrotic factor TGFβ, S100A8/A9 led to irreversible damage and cell death. Therefore, our data suggest that S100A8/A9 mediates the development of renal fibrosis, possibly through a direct effect on dedifferentiation and apoptosis in TECs.

Chapter 4 introduces the reader to a different inflammatory mediator: Triggering Receptor expressed on myeloid cells-1 (TREM-1). Herein, we extensively reviewed

TREM-1 function, from biological relevance to therapeutic intervention, solely in sterile inflammatory disorders.

Our contribution in this field begins with **Chapter 5**, where we investigated the role of TREM-1 in an experimental model of IR-induced AKI and in human renal transplantation. WT mice were subjected to bilateral IR injury and sacrificed 24 hours later to study the acute inflammatory response. We found that during IR injury, there is an infiltration of cells transcribing TREM-1 in the renal interstitium. Moreover, both the TREM-1 receptor and its soluble protein are increased in tissue lysates and plasma. Monocytic TREM-1 expression was also found to be increased. Taken together, this suggests that TREM-1 may be involved in the amplification of the inflammatory signals driving the development of IR injury. To further evaluate this hypothesis, we treated the mice with different TREM-1 inhibitors, which were previously shown to modulate TREM-1-induced inflammation and prevented tissue dysfunction. Despite downregulation of the TREM-1 pathway, the approaches we used failed to protect the kidney from inflammation and damage. Additionally, we evaluated whether Single Nucleotide Variants (SNVs) in the TREM-1 gene were associated with any pathological outcomes after kidney transplantation. In line with the findings from the preclinical model, we observed that neither donor nor recipient carriers of the TREM-1 gene variant p.Thr25Ser (heterozygous and non synonymous) were associated with delayed graft function, rejection or graft failure. From this study, it appears that TREM-1 does not play a prominent role in the kidney's acute response to hypoxic damage.

Since PRRs may also be involved in kidney regeneration after damage, we sought to carry out an in depth study into the role of TREM-1 in the repair phase of IR injury. Therefore, in **Chapter 6,** we subjected WT and TREM1/3 KO animals to renal IR and monitored the mice to study the injury, repair and resolutive phase following hypoxic damage. Herein, we confirmed that TREM-1 pathway activation is dispensable during the acute phase of injury, as WT and TREM1/3 KO mice display similar degrees of renal inflammation and damage following 24 hours of reperfusion. To our surprise, we observed increased mortality in TREM1/3KO animals during the repair phase. By switching to a milder model of renal damage we were able to show that TREM1/3 KO mice suffer from maladaptive repair with progression to CKD. In the absence of TREM1/3, in particular, we observed persistent tubular damage and interstitial fibrosis, as shown by increased macrophage infiltration, accumulation of α-SMA+ cells and collagen deposition. Additionally, TREM1/3-deficient mice failed to regenerate TECs, due to the development of tubular senescence and the associated reduction in proliferation. By means of *in vitro*-simulated IR experiments, we were able to show that TREM-1 is expressed on TECs after hypoxic damage, suggesting a direct effect of TREM-1 in the tubular response to injury. A possible mechanism driving

the failed tubular regeneration after IR could be found in the fact that at steady state TREM1/3 KO TECs already experience a cell cycle blockade, specifically in the G2/M phase. This growth arrest was possibly related to major differences we detected in the anabolic and metabolic pathways between WT and TREM1/3 deficient TECs, particularly those related to mitochondrial antioxidant levels, suggesting an increase in oxidative stress in the absence of TREM1/3. Indeed, TREM1/3-deficient TECs display an altered mitochondrial homeostasis, with disrupted morphology, increased ROS accumulation, mitochondria depolarization and impaired capacity for energy production. Additionally, this resulted in an increased expression of pro-inflammatory and fibrotic mediators by TECs. This phenotype worsened when cells were exposed to *in vitro*-simulated ischemia. The increase in cellular senescence resulted in a permanent growth arrest in TECs , which led to an altered wound healing response. Thus, we identified a novel role for TREM-1 in tubular epithelial cells, which preserves mitochondrial integrity and confers a metabolic advantage for TECs to progress into the cell cycle and proliferate. In summary, TREM-1 fosters tubular regeneration and limits maladaptive repair post-AKI.

Since maladaptive repair led to fibrosis, in **Chapter 7,** we evaluated the specific role of TREM-1 and its adaptor molecule DAP12, in the chronic model of obstructive nephropathy, which is the classic model for renal fibrosis. Herein, we show that in patients with obstructive hydronephrosis, TREM-1 was detected in interstitial cells, but was absent in protocolar renal biopsies of transplanted patients with stable graft function. In the experimental model, we subjected WT, TREM1/3 KO and DAP12 KO animals to permanent UUO and analyzed the animals at different time points post-obstruction. Although UUO induces the transcription of TREM-1 and DAP12 by interstitial cells, it appears that DAP12, partly through TREM1/3, mediates renal inflammation after UUO, but that both play a dispensable role in the development of renal fibrosis. Indeed, myofibroblast accumulation and collagen deposition were similar between the experimental groups. However, in the absence of TREM1/3 and DAP12, we observed increased tubular damage and edema in the early stages after obstruction, suggesting a possible role for TREM1/3 and DAP12 in tubular integrity.

Nederlandse samenvatting

De incidentie van nierziekten is aanzienlijk toegenomen en is wereldwijd een belangrijke oorzaak van morbiditeit en mortaliteit. Gerichte behandelingen voor patiënten met nierziekten zijn nog steeds niet beschikbaar. De huidige therapieën, die alleen ondersteunend zijn, bestaan uit dialyse en niertransplantatie. Een beter begrip van de pathogenese van nierziekten is nodig voor de ontwikkeling van nieuwe behandelingsmogelijkheden. Het aangeboren immuunsysteem vertegenwoordigt de eerste verdedigingslinie tegen binnendringende pathogenen, maar speelt ook een cruciale rol bij renale beschadiging. Eiwitten die vrijkomen door beschadigde- of inflammatoire-cellen, de zogenaamde Damage Associated Molecular Patterns (DAMPs), werken als een ligand voor Pattern Recognition Receptors (PRRs) en initiëren een ontstekingsreactie. Dit resulteert in een klaring van binnenvallende pathogenen en weefselherstel. Ontstekingscellen zijn de belangrijkste spelers in de meeste inflammatoire processen.

Het gevaars-eiwit S100A8/A9 en de aangeboren immuun-receptor TREM-1 worden tot expressie gebracht door neutrofielen en monocyten/ macrofagen; beide kunnen ontstekingssignalen opwekken of versterken via PRRs. Dit proefschrift richt zich op de rol van het gevaars-eiwit S100A8/A9 en de activerende immuun-receptor TREM-1 in preklinische modellen van acute nierschade (AKI) en chronische nierziekte (CKD).
In **hoofdstuk 1** geven we een kort overzicht van de renale homeostase en ziekten, samen met een gedetailleerde beschrijving van factoren die betrokken zijn bij de aangeboren immuunrespons, dat de belangrijkste focus is voor het onderzoek beschreven in dit proefschrift.

Hoofdstuk 2 beschrijft de bijdrage van S100A8/A9 aan renale ischemie/reperfusie (IR)-geïnduceerde AKI, hierin wordt de rol die gevaars-eiwitten spelen bij renaal herstel na de AKI onderzocht. We onderwierpen zowel wildtype als S100A9 KO-dieren aan een bilaterale afklemming van de nierslagader, gevolgd door verschillende dagen van reperfusie om de verschillende fasen van renale beschadiging en herstel te bestuderen. We hebben waargenomen dat de expressie van S100A8/A9 in de nier was verhoogd tijdens IR-letsel, met name 24 uur na ischemie. Dit kan hoogstwaarschijnlijk worden toegeschreven aan de infiltratie van granulocyten die hoge levels van S100A8/A9 tot expressie brengen. Ondanks de verhoogde expressie van het eiwit 1 dag na IR, vertoonden S100A9 KO-dieren op dit tijdstip geen belangrijke veranderingen in renale inflammatie of veranderde expressie van renale schademarkers. We hebben echter vastgesteld dat S100A8/A9 cruciaal is in het beperken van de maladaptieve herstel na

AKI. Tijdens de regeneratieve-fase vertoonden S100A9 KO-muizen inderdaad tekenen van nier disfunctie in combinatie met verhoogde expressie van tubulaire schade markers, wat is geassocieerd met een verhoogde productie van chemokinen en cytokinen in de nier. Ten slotte laten we zien dat S100A9 KO-dieren nierfibrose ontwikkelen, zoals blijkt uit een verhoogde collageendepositie en TGFβ-productie. Het maladaptieve herstel-fenotype waargenomen in de S100A9 KO-dieren kan volgens ons worden toegeschreven aan een veranderde macrofaagfunctie. Gezien de rol die macrofagen spelen bij weefselherstel is dit inderdaad goed mogelijk,. S100A9 KO-macrofagen laten in vivo een afwijkend M2-achtig fenotype zien, door verhoogde transcriptie van alternatieve M2-macrofaagactiveringsmarkers, zoals Arg-l, MGL1 en IRF5. Door middel van in vitro experimenten bevestigden we dat na M2-skewing met IL4- en IL13-cytokines S100A9 KO-macrofagen een fenotype vertoonden dat vergelijkbaar is met het waargenomen in vivo fenotype. Mogelijk kan tijdens de reparatiefase buitensporige M2-polarisatie, welke vaak gepaard gaat met het vrijkomen van pro-fibrotische factoren, de onjuiste reactie op genezing verder ontwikkelen, wat leidt tot verlittekening van het weefsel. S100A8/A9 is door het beheersen van een overmatige M2-macrofaagpolarisatie en het voorkomen van nierfibrose dus van cruciaal belang bij het herstel van de nier na ischemie.

Het is bekend dat macrofagen zich ophopen in de nier tijdens nierfibrose en mogelijk ook een rol spelen bij weefselafbraak. Aangezien we hebben waargenomen dat S100A9 KO-dieren verhoogde fibrose vertonen bij AKI als gevolg van overmatige M2-macrofaagpolarisatie, onderzochten we in **hoofdstuk 3** de rol van S100A8/A9 tijdens de ontwikkeling van renale fibrose. In dit hoofdstuk gebruikten we het eenzijdige ureter obstructie (UUO) als chronisch model voor renale beschadiging, wat leidt tot de ontwikkeling van ernstige tubulo-interstitiële fibrose in WT en S100A9 KO dieren. Gedurende het UUO verloop in WT nieren werd een toename in de aanwezigheid van intra-renale S100A8/A9-positieve cellen die ook de granulocyt activatiemarker Ly6G tot expressie brengen waargenomen. De infiltratie van deze ontstekingscellen verschilde echter niet tussen WT en S100A9 KO-dieren. Het expressieprofiel van S100A8/A9 in het preklinische UUO model was overeenkomend met het profiel dat werd waargenomen bij patiënten met obstructieve hydronefrose. In tegenstelling tot wat eerder is aangetoond, vonden wij door verminderde infiltratie van myofibroblasten en collageenafzetting, dat S100A9 KO-dieren beschermd zijn tegen nierfibrose. We vonden dat de reductie in fibrose, waargenomen bij KO dieren die chronische schade hadden opgelopen, gerelateerd was aan het behoud van tubulaire epitheliale cel (TEC) integriteit. S100A9 KO-dieren vertoonden namelijk verminderde tubulaire apoptose en activering van belangrijke epitheliale-mesenchymale transitie parameters. In overeenstemming met deze bevindingen hebben we waargenomen dat stimulatie van TECs met recombinant

S100A8/A9 eiwit stopzetting van de celcyclus en tekenen van dedifferentiatie induceert, waaronder verminderde expressie van cel vermenigvuldiging en adhesiemoleculen die essentieel zijn bij het ondersteunen van de renale tubuli structuur en functie. Stimulatie met de pro-fibrotische factor TGFβ in aanwezigheid van recombinant S100A8/A9 eiwit leidde tot onomkeerbare schade en celdood van TECs. Daarom suggereert onze studie dat S100A8/A9 de ontwikkeling van renale fibrose medieert, mogelijk door een direct effect op TEC dedifferentiatie en apoptose.

Hoofdstuk 4 introduceert een andere inflammatoire mediator: Triggering Receptor expressed on myeloid cells-1 (TREM-1). In dit hoodstuk hebben we uitvoerig de functie van TREM-1 in uitsluitend steriele ontstekingsaandoeningen, van biologische relevantie tot therapeutische interventie, gereviewd.
Onze bijdrage op dit gebied begint met **hoofdstuk 5**, waarin we de rol van TREM-1 in een experimenteel model van IR-geïnduceerde AKI en bij menselijke niertransplantatie hebben onderzocht. WT muizen werden onderworpen aan bilaterale IR en 24 uur later opgeofferd om de acute ontstekingsreactie te bestuderen. We vonden dat er tijdens IR-letsel een infiltratie ontstaat van cellen die TREM-1 tot expressie brengen in het renale interstitium. Bovendien zijn zowel de TREM-1 receptor als het TREM-1 oplosbare eiwit verhoogd aanwezig in zowel nier weefsel lysaten als plasma. De TREM-1 expressie door monocyten bleek ook te zijn toegenomen. Dit suggereert dat TREM-1 mogelijk betrokken is bij de amplificatie van de ontstekingssignalen die de ontwikkeling van IR-letsel stimuleren. Om deze hypothese verder te evalueren, hebben we de muizen behandeld met verschillende TREM-1 remmers waarvan eerder is aangetoond dat ze TREM-1 geïnduceerde ontsteking moduleren en weefseldisfunctie voorkomen. Ondanks de downregulatie van de TREM-1 pathway, bleek deze interventie de nier niet te beschermen tegen ontsteking en schade. Bovendien onderzochten we of Single Nucleotide Variants (SNV's) in het TREM-1 gen geassocieerd zijn met pathologische ontwikkelingen na niertransplantatie. In overeenstemming met de bevindingen van het preklinische model, hebben we waargenomen dat noch donor noch ontvangende dragers van de TREM-1 genvariant p.Thr25Ser (heterozygoot en niet-synoniem) worden geassocieerd met een vertraagde niertransplantaat functie, afstoting of transplantaat falen. Uit deze studie blijkt dat TREM-1 geen prominente rol speelt in de reactie van de nieren op acute hypoxische schade.

Aangezien PRRs mogelijk ook betrokken zijn bij renale regeneratie na beschadiging, hebben we de rol van TREM-1 in de reparatiefase van IR-letsel te onderzoeken. Daarom hebben we in **hoofdstuk 6** WT en TREM1/3 KO dieren onderworpen aan renale IR en de muizen gevolgd om de letsel-, herstel- en resolutiefase na hypoxische schade te

bestuderen. Hiermee hebben we bevestigd dat activering van de TREM-1-pathway overbodig is tijdens de acute fase van de schade, aangezien WT en TREM1/3 KO muizen na 24 uur reperfusie een vergelijkbare graad van renale-ontsteking en -beschadiging vertonen. Tot onze verbazing constateerden we een verhoogde mortaliteit bij TREM1/3 KO dieren tijdens de herstelfase. Door over te schakelen op een milder model van acute renale beschadiging konden we aantonen dat TREM1/3 KO muizen een maladaptieve repair ondergaan met progressie naar CKD. In afwezigheid van met name TREM1/3, observeerden we aanhoudende tubulaire schade en interstitiële fibrose, zoals aangetoond door de verhoogde infiltratie van macrofagen, accumulatie van α-SMA positieve cellen en collageenafzetting. Bovendien werd er geen regeneratie gezien van TECs in TREM1/3 deficiënte muizen, vanwege de ontwikkeling van tubulaire senescence en de daarmee gepaard gaande vermindering in proliferatie. Door middel van in vitro gesimuleerde IR-experimenten konden we aantonen dat TREM-1 na hypoxische schade op TECs tot expressie wordt gebracht, wat duidt op een direct effect van TREM-1 in de tubulaire respons op letsel. Een mogelijk mechanisme dat de gefaalde tubulaire regeneratie na IR kan verklaren, kan worden gevonden in het feit dat TREM1/3 KO TECs op basaal niveau al een blokkade van de celcyclus ervaren, met name in de G2/M-fase. Deze groeistop is mogelijk gerelateerd aan de grote verschillen die we detecteerden in de anabole en metabole pathways tussen WT en TREM1/3 deficiënte TECs, met name de dominante aanwezigheid van pathways gerelateerd aan mitochondriale antioxidant niveaus in TREM1/3 deficiënte TECs duidt op een toename van oxidatieve stress in afwezigheid van TREM1/3. TREM1/3 deficiënte TECs vertoonden inderdaad een veranderde mitochondriale homeostase, met een verstoorde mitochondriale morfologie, verhoogde ROS-accumulatie, depolarisatie van mitochondria en een verminderde energie producerende capaciteit. Bovendien resulteerde dit in een verhoogde expressie van pro-inflammatoire en fibrotische mediatoren door TECs. Dit fenotype verslechterde toen cellen werden blootgesteld aan ischemie. De toename in cellulaire senescence resulteerde in een permanente groei-arrest van TECs, wat leidde tot een veranderde wondgenezing. We identificeerden hiermee een nieuwe rol voor TREM-1 in tubulaire epitheliale cellen, waarbij TREM-1 zorgt voor behoud van de mitochondriale integriteit en een metabool voordeel geeft aan TECs wat nodig is voor het verloop van de celcyclus en proliferatie. Samengevat, TREM-1 bevordert tubulaire regeneratie en beperkt maladaptief herstel na AKI.

Omdat maladaptief herstel leidde tot fibrosis, in **hoofdstuk 7**, we evalueerden de specifieke rol van TREM-1 en zijn adaptormolecuul DAP12, in het chronische model van obstructieve nefropathie en het klassieke model voor nierfibrose. Hierin laten we zien dat bij patiënten met obstructieve hydronefrose TREM-1 werd gedetecteerd in interstitiële

cellen, maar afwezig was in protocolaire nierbiopten van getransplanteerde patiënten met een stabiele niertransplantaat functie. In het experimentele model stelden we WT, TREM1/3 KO en DAP12 KO-dieren bloot aan UUO en analyseerden de nieren van deze dieren op verschillende tijdstippen na obstructie. Hoewel UUO de transcriptie van TREM-1 en DAP12 door interstitiële cellen induceert en DAP12, deels via TREM 1/3, invloed uitoefent op de renale inflammatierespons, vonden we dat zowel TREM-1 als DAP12 geen leidende rol spelen in de ontwikkeling van nierfibrose. De accumulatie van myofibroblasten en de collageenafzetting waren immers vergelijkbaar tussen de experimentele groepen. In de afwezigheid van TREM1/3 en DAP12 hebben we echter in de vroege stadia na obstructie wel verhoogde tubulaire schade en oedeem waargenomen, wat duidt op een mogelijke rol voor TREM1/3 en DAP12 in het behoudt van de tubulaire integriteit.

Riassunto

L'incidenza delle malattie renali è aumentata in modo significativo ed è una delle principali cause di morbidità e mortalità in tutto il mondo. Una specifica terapia per i pazienti affetti da malattie renali non è disponibile e le terapie attuali, che sono solo di supporto, consistono nella dialisi e nel trapianto renale. Per sviluppare nuove opportunità di trattamento e' necessaria una maggiore comprensione della patogenesi delle malattie renali. Il sistema immunitario innato che possediamo, rappresenta la prima linea di difesa contro i patogeni, ma svolge anche un ruolo cruciale nel danno tissutale renale. Le proteine associate al danno che vengono rilasciate da cellule danneggiate o infiammatorie, i cosiddetti Damaged associated molecular pattern (DAMP), agiscono da ligando per i Pattern Recognition receptor (PRR) dando inizio così a una reazione infiammatoria. Questo si traduce in una eliminazione dei patogeni e il conseguente riparo del danno tissutale causato da quest'ultimi.
Le cellule infiammatorie sono i principali protagonisti nella maggior parte in questo processo. La proteina S100A8/A9 e il recettore TREM-1 sono espressi da neutrofili e monociti/macrofagi; entrambi sono in grado di dare inizio o amplificare la risposta infiammatoria attraverso altri PRR. L'obiettivo di questa tesi è stato quello di studiare il ruolo di S100A8/A9 e TREM-1 in modelli preclinici di danno renale acuto (AKI) e malattia renale cronica (CKD).

Nel **Capitolo 1** viene fornita una breve panoramica della funzionalita' renale e delle varie malattie che sono state oggetto di questa tesi. Il **Capitolo 2** invece, descrive il contributo di S100A8/A9 nel modello di AKI indotto dall' ischemia/riperfusione (IR) renale ed esplora il ruolo significativo che questa proteina svolge nella rigenerazione renale dopo il danno causato da IR. Infatti abbiamo sottoposto sia topi Wild Type (WT) che animali senza S100A8/A9 (S100A9 KO) all'occlusione delle arterie renali per diversi minuti, che ha generato una carenza d'ossigeno al rene dovuta alla ridotta perfusione sanguigna (=ischemia). Dopodiche'abbiamo rilasciato l'occlusione in modo da ristabilire la perfusione (=riperfusione) e abbiamo monitarato i topi per diversi giorni per valutare l'effetto sul rene dettato dall'assenza di S100A8/A9. Nei topi WT e' stato osservato che l'espressione di S100A8/A9 aumenta nel rene durante IR, in particolare 24 ore dopo l'ischemia. Questo può essere attribuito molto probabilmente all'infiltrazione dei granulociti che esprimono alti livelli di S100A8/A9. Nonostante l'aumentata espressione della proteina un giorno dopo l'IR, gli animali S100A9 KO non hanno mostrato grossi cambiamenti nell'infiammazione renale ne' tantomeno hanno mostrato segni di danno. Tuttavia, abbiamo scoperto che S100A8/A9 è fondamentale nel riparo del danno renale dopo IR. Infatti, durante la fase di riparo, i topi S100A9 KO hanno mostrato segni di

disfunzione renale e un aumento d'espressione di segnali di danno provenienti dalle cellule tubulari renali, che era inoltre associato ad un aumento della produzione di chemochine e citochine, che richiamano cellule infiammatorie nel rene. Infine, abbiamo notato che gli animali S100A9 KO hanno sviluppato fibrosi renale, come evidenziato dall'aumento della deposizione di collagene e della produzione della proteina fibrotica TGFβ.
E' stato poi riscontrato che la fibrosi renale fosse dovuta a una funzione alterata dei macrofagi che avevano i topi S100A9 KO. Questo è plausibile, considerato che i macrofagi giocano un ruolo fondamentale nel riparo dei tessuti, specialmente dopo IR. I macrofagi S100A9 KO hanno un fenotipo M2 alterato, come è stato dimostrato dall'aumentata trascrizione di geni coinvolti nell'attivazione M2 dei macrofagi, ovvero Arg1, MGL1 e IRF5. Attraverso esperimenti *in vitro*, abbiamo confermato che dopo stimolazione con le citochine IL4 e IL13, i macrofagi S100A9 KO hanno mostrato un fenotipo simile a quello osservato *in vivo*, ovvero alterata attivazione M2. Probabilmente, durante la fase di riparo, un'eccessiva polarizzazione M2, che è spesso associata al rilascio di fattori pro-fibrotici, può ulteriormente alimentare il processo fibrotico, portando così alla formazione di cicatrici. Pertanto, S100A8/A9 è coinvolto in modo cruciale nel riparo renale dopo ischemia, controllando che la polarizzazione dei macrofagi M2 avvenga in maniera controllata, cosi' da prevenire la fibrosi renale.

E' risaputo che i macrofagi si accumulano nel rene durante la fibrosi renale e possono anche avere un ruolo nella degenerazione dei tessuti. Così, siccome abbiamo osservato che gli animali S100A9 KO mostrano una fibrosi nel modello di danno acuto (AKI) a causa dell'eccessiva polarizzazione dei macrofagi M2, nel **Capitolo 3** abbiamo ulteriormente analizzato il ruolo di S100A8/A9 nello sviluppo della fibrosi renale. In questo capitolo abbiamo usato l'ostruzione ureterale unilaterale (UUO) negli animali WT e S100A9 KO, come modello cronico di danno che conduce allo sviluppo di una grave fibrosi renale. Durante lo sviluppo della fibrosi renale, le cellule positive per S100A8/A9 si accumulano nel rene e co-esprimono il marcatore di attivazione dei granulociti Ly6G; quindi sembrano essere dei granulociti. Tuttavia, l'infiltrazione di queste cellule infiammatorie non differisce tra gli animali WT e S100A9 KO. Il profilo di espressione di S100A8/A9 nel modello preclinico era analogo a quello osservato nei pazienti con idronefrosi. In contrasto con cio'che abbiamo riscontrato nel modello di danno renale acuto, gli animali S100A9 KO hanno sviluppato un attenuata fibrosi renale, come dimostrato dalla diminuzione dell'infiltrazione di miofibroblasti e della deposizione di collagene. E' stato scoperto che la ridotta fibrosi, osservata negli animali KO che avevano subito un danno cronico, era correlata all'integrita' delle cellule epiteliali tubulari (TECs). Gli animali S100A9 KO hanno mostrato una diminuzione dell'apoptosi tubulare e dei processi di

transizione epiteliale-mesenchimale. In linea con questi risultati, è stato osservato che la stimolazione delle TECs con la proteina ricombinante S100A8/A9 induceva l'arresto del ciclo cellulare e portava a manifestare segni di dedifferenziazione epiteliale, inclusa la diminuita espressione delle proteine della giunzione cellulare e delle molecole di adesione, che sono essenziali nel mantenere la struttura e la funzione tubulare. Inoltre S100A8/A9, insieme al fattore profibrotico TGFβ, ha portato a un danno irreversibile e morte cellulare attraverso apoptosi nelle TECs. Pertanto, questi dati suggeriscono che S100A8/A9 e' coinvolto nello sviluppo della fibrosi renale, probabilmente attraverso un effetto diretto sulla dedifferenziazione e apoptosi delle TECs.

Il **capitolo 4** introduce al lettore un altro mediatore dell'infiammazione: il recettore di attivazione espresso su cellule mieloidi-1 (TREM-1). In questo capitolo viene ampiamente riassunta la funzione di TREM-1, a partire dalla rilevanza biologica fino all'intervento terapeutico, esclusivamente in malattie infiammatori che non coinvolgono patogeni.

Il nostro contributo in questo campo inizia con il **capitolo 5**, in cui abbiamo studiato il ruolo di TREM-1 nel modello sperimentale di AKI indotto da IR e nel trapianto renale umano. I topi WT sono stati sottoposti a IR e sacrificati 24 ore dopo, col fine di studiare la risposta infiammatoria acuta. Abbiamo scoperto che durante il danno da IR, si verifica un'infiltrazione di cellule che esprimono TREM-1 nell'interstizio renale. Inoltre, sia il recettore TREM-1 che la proteina solubile risultano aumentati nei rene e nel plasma dei topi. Anche l'espressione monocitica di TREM-1 è risultata aumentata. Ciò suggerisce che TREM-1 potrebbe essere coinvolto nell'amplificazione dei segnali infiammatori che guidano lo sviluppo del danno da IR. Per valutare ulteriormente questa ipotesi, abbiamo trattato i topi con diversi inibitori di TREM-1, che in precedenza si erano dimostrati efficaci nell'inibire l'azione dannosa legata all'eccessiva infiammazione da TREM-1 e di prevenire la disfunzione renale. Nonostante gli inibitori hanno ridotto l'espressione di TREM-1 e delle molecole associate ad esso, gli approcci usati non sono riusciti a proteggere il rene dall'infiammazione e dai danni legati all'IR. Inoltre, abbiamo valutato se le Varianti Nucleotidiche Singole (SNVs) nel gene di TREM-1 fossero associate ad eventuali esiti patologici dopo un trapianto di rene. In linea con i risultati del modello preclinico, abbiamo osservato che né i donatori né i trapiantati che possedevano la specifica variante genica p.Thr25Ser (eterozigote e non sinonimo) nel gene di TREM-1 mostravano alcun esito patologico a lungo termine dopo il trapianto. Da questo studio appare che TREM-1 non svolge un ruolo rilevante nella risposta acuta del rene al danno ipossico.

Ma poiché i PRR possono anche essere coinvolti nella rigenerazione dei reni dopo il danno, abbiamo cercato di effettuare uno studio approfondito sul ruolo di TREM-1 nella

fase di riparo dal danno causato da IR. Pertanto, nel **Capitolo 6**, abbiamo sottoposto gli animali WT e TREM1/3 KO a IR renale e in più i topi sono stati monitorati per studiare la fase del danno, del riparo e della rigenerazione renale dopo il danno ipossico. A questo punto, abbiamo confermato che l'attivazione di TREM-1 non ha alcun effetto durante la fase acuta dopo danno ischemico, in quanto i topi WT e TREM1/3 KO hanno mostrato gradi simili di infiammazione e di danno renale dopo 24 ore di riperfusione. Con sorpresa, abbiamo osservato un aumento della mortalità negli animali TREM1/3 KO durante la fase di riparo. Passando a un modello più lieve di danno siamo stati in grado di dimostrare che i topi TREM1/3 KO soffrono di un alterata fase di riparo che porta allo sviluppo del danno cronico, a partire da quello acuto. In assenza di TREM1/3, in particolare, abbiamo osservato un danno persistente alle cellule tubulari e una fibrosi interstiziale, come dimostrato da una maggiore infiltrazione dei macrofagi, da un accumulo di fibroblasti e dalla deposizione di collagene. Inoltre, i topi con deficit di TREM1/3 non sono riusciti a rigenerare le cellule tubulari, a causa dello sviluppo della senescenza tubulare e della ridotta proliferazione. Attraverso alcuni esperimenti di IR *in vitro*, siamo stati in grado di dimostrare che TREM-1 viene espresso su TECs dopo il danno ipossico, suggerendo un effetto diretto di TREM-1 nella rigenerazione tubulare. Un possibile meccanismo che potrebbe essere alla base della fallita rigenerazione tubulare dopo IR, può essere trovato nel fatto che allo stato stazionario le cellule tubulari che non possiedono TREM1/3 già sperimentano un blocco del ciclo cellulare, in particolare nella fase G2/M. Questo arresto della crescita e' probabilmente correlato al diminuito metabolismo riscontrato nelle TECs che non hanno TREM1/3, in particolare relativi ai livelli di antiossidanti, suggerendo un aumento di stress ossidativo in assenza di TREM1/3. Infatti, queste TECs senza TREM1/3 mostrano alterata omeostasi mitocondriale, con morfologia perturbata, un aumento dell'accumulo di ROS, depolarizzazione dei mitocondri e ridotta capacità di produrre energia. Inoltre, questo ha comportato una maggiore espressione di mediatori pro-infiammatori e fibrotici in queste TECs. Questo fenotipo risulta peggiorato quando le cellule vengono esposte all'IR *in vitro*. Infatti, dopo il danno ischemico queste cellule mostrano un arresto permanente della proliferazione cellulare, definito come senescenza, che ha portato ad una alterata risposta al riparo del danno. Pertanto, abbiamo identificato un nuovo ruolo per TREM-1 nelle cellule epiteliali tubulari, che preserva l'integrità mitocondriale e conferisce un vantaggio metabolico affinché le TEC progrediscano nel ciclo cellulare e proliferino. Per concludere: TREM-1 favorisce la rigenerazione tubulare e limita il danno maladattivo che porta alla progression verso la fibrosi renale.

Visto cio' nel **Capitolo 7** abbiamo valutato il ruolo specifico di TREM-1 e della sua molecola adattatrice DAP12, nel modello cronico di nefropatia ostruttiva unilaterale (UUO), che è il modello classico per la fibrosi renale. Qui, mostriamo che nei pazienti con idronefrosi,

TREM-1 è stato rilevato nelle cellule interstiziali, ma era assente nelle biopsie renali protocolari di pazienti trapiantati con stabile funzione renale. Nel modello sperimentale, abbiamo sottoposto gli animali WT, TREM1/3 KO e DAP12 KO a UUO permanente e abbiamo analizzato gli animali in diversi tempi in fase post-ostruzione. Sebbene UUO porta ad un aumento di espressione di TREM-1 e DAP12 da parte delle cellule interstiziali, sembra che DAP12, in parte attraverso TREM1/3, tende a mediare l'infiammazione renale dopo UUO, ma che entrambi non svolgano un ruolo decisivo nello sviluppo della fibrosi renale. Infatti, l'accumulo di miofibroblasti e i depositi di collagene risultavano simili tra i diversi gruppi di animali. Tuttavia, in assenza di TREM1/3 e DAP12, abbiamo osservato un aumento del danno tubulare e dell'edema nelle prime fasi dopo l'ostruzione, suggerendo un possibile ruolo per TREM1/3 e DAP12 nell'integrità tubulare.

PhD portfolio

Alessandra Tammaro

PhD period: November 2012-2017

PhD supervisor: Prof. dr. S. Florquin

Co-supervisors: dr. M.C. Dessing

dr. J.C. Leemans

	Year
Courses	
- Laboratory Animal Science (Art.9 certification)	2013
- Basic Laboratory safety	2013
- The AMC world of science	2013
- DNA technology	2013
- Scientific writing for publication	2013
- Advanced immunology	2015
Seminars, workshops and master classes	
- Masterclass by Prof. Roy Bloom	2017
- Weekly departments and journal club meetings	2012-17
- APROVE symposium "Sexy science"	2013
- APROVE symposium "Making money in science"	2014
Conferences and presentation	
- European Congress Immunology (ECI 2018), Amsterdam, The Netherlands (oral presentation)	2018
- Keystone symposia, Injury inflammation and fibrosis, Snow bird, Utah, USA (poster presentation)	2017
- Amsterdam infection and immunity retreat, Heemskerk, The Netherlands (oral presentation)	2017
- Dutch symposia of Nephrology, Veldhoven, the Netherlands (oral presentation)	2016
- ENII summer school in immunology, Porto Cervo, Italy (poster and oral presentation)	2016
- International retreat of PhD students in Immunology, Naples, Italy (poster and oral presentation)	2016
- Dutch fall symposia of Nephrology, Lunteren, the Netherlands (poster and oral presentation)	2016
- Toll 2015, Marbella, Spain	2015
- Dutch symposia of Nephrology, Veldhoven, the Netherlands (poster presentation)	2015
- American Association of immunological society 2015- New Orleans (LA) (poster presentation)	2015
- American Society of nephrology- Kidney week, Philadelphia, PA (USA)	2014
- Fall Symposia of Nephrology, Utrecht, the Netherlands	2013
- Dutch symposia of Nephrology, Veldhoven, the Netherlands (poster presentation)	2013
- Platform Aio Nephrology (PLAN) day, Rotterdam, the Netherlands	2013

Lecturing	
- Lecture on Innate immune signalling in acute and chronic renal injury	2017
Tutoring, Mentoring/Supervising	
- Research projects (student): Role of S100A8/A9 in obstructive nephropathy	2016
- Research projects (Erasmus student): Role of TREM-1 in IR-induced AKI	2016
- Research projects (research collaboration): Role of TREM-1 in tubular epithelial cell senescence.	2017
Parameters of Esteem	
- Amsterdam infection and immunity institute: travel award	2017
- Spinoza grant (University of Amsterdam): travel grant	2017
- European network of immunology society: travel grant	2016
- SIICA (italian society of immunology): travel grant	2016
- European Society for Organ Transplantation- Basic science travel grant	2015
- European Federation of Immunological Societies-IL world fellowship visiting grant	2014
- Dutch Kidney Foundation: Kolff PhD/Post doc fellowship visiting grant	2014
Peer reviews	
- 1 article for journal of Crohn's and colitis (impact factor 5.9)	2017
- 1 article for Pathology research and practice (Impact factor 1.54)	2017
Additional Activities	
- Co-organizer of the Promovendi Netwerk Nederland (PNN)'s "National PhD day"	2016

Publications

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A. Tammaro, J. Kers, D. Emal, I. Stroo, G. J. D. Teske, L. M. Butter, N. Claessen, J. Damman, M. Derive, G. Navis, S. Florquin, J. C. Leemans, and M. C. Dessing, "Effect of TREM-1 blockade and single nucleotide variants in experimental renal injury and kidney transplantation." *Sci Rep*. 2016 Dec 8;6:38275

A. Tammaro, M. Derive, S. Gibot, J. C. Leemans, S. Florquin, and M. C. Dessing, "TREM-1 and its potential ligands in non-infectious diseases: from biology to clinical perspectives." *Pharmacol Ther.* 2017 Sep;177:81-95.

A. Tammaro, S. Florquin, M. Brok, N. Claessen, L. M. Butter, G. J. D. Teske, O. J. de Boer, T. Vogl, J. C. Leemans, and M. C. Dessing, "S100A8/A9 promotes parenchymal damage and renal fibrosis in obstructive nephropathy." *Clin. Exp. Immunol*., 2018.

About the author

Alessandra Tammaro, daughter of Stefania and Pietro, was born on April 27th 1987 in Naples, Italy. Since high school she manifested a passion for biology that brought her in march 2010 to receive the bachelor diploma in biotechnology, from the University of Naples. Afterwards, she moved to London for several months to improve the english language and obtained several certifications. She continued with master studies in medical biotechnology in Naples, where she carried out a scientific research at Francesco Beguinot's laboratory (National Research Council Institute, Naples). Here, she investigated vascular complications associated with type 2 diabetes. Meanwhile, having received an Erasmus scholarship, she moved to Amsterdam for an additional research in Carlie de Vries's laboratory (Medical Biochemistry department, AMC, university of Amsterdam), where she studied the effect of a cysteine protease inhibitor in the pathology of aorta aneurism, under the supervision of Dr. V. de Waard. In 2012 she obtained her master degree *cum laude* from the University of Naples. In the same year she returned to Amsterdam to begin her PhD program at the department of pathology at AMC, under the supervision of Prof. Dr. Sandrine Florquin, Dr. J.C. Leemans and Dr. M.C. Dessing. In 2014 she was awarded two fellowships for a research collaboration with Prof. Dr. Marco Colonna, that brought her to St. Louis (Missouri, USA) to work at the department of immunology of the prestigious Washington University School of Medicine. She completed her doctoral studies at AMC and the results of her PhD research are the subject of this thesis. On November 2017, together with her partner Jorge, became parents of Angelo Leonardo. Alessandra will continue her research career in kidney diseases at the department of pathology of the Amsterdam UMC, in the group of Prof. Dr. S. Florquin.

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