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Targeting immunosenescence to prevent kidney injury

Snigdha Nitin Rao

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THÈSE

**En vue de l'obtention du
DOCTORAT DE L'UNIVERSITÉ DE TOULOUSE**

Délivré par l'Université Toulouse 3 - Paul Sabatier

Cotutelle internationale: Université autonome de Madrid

Présentée et soutenue par

Snigdha Nitin RAO

Le 28 novembre 2023

**Ciblage de l'immuno-sénescence pour prévenir les maladies
rénales**

Ecole doctorale : BSB - Biologie, Santé, Biotechnologies

Spécialité : MALADIES METABOLIQUES ET CARDIOVASCULAIRES

Unité de recherche :

I2MC - Institut des Maladies Métaboliques et Cardiovasculaires

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Résumé

L'insuffisance rénale aiguë (IRA) est une pathologie fréquente et grave, associée à un risque de développer une maladie rénale chronique (MRC). Bien que l'incidence et la morbi-mortalité de l'insuffisance rénale aiguë soient significativement plus élevées chez les personnes âgées que chez les patients plus jeunes, la susceptibilité élevée de la population âgée à l'insuffisance rénale aiguë est la connue. L'immunosénescence (IS) (désignant les changements du système immunitaire induits par le vieillissement) pourrait être le dénominateur commun entre l'IRA, l'IRC et le vieillissement. Nous avons émis l'hypothèse novatrice que l'IS pourrait également survenir au cours de l'IRA et que sa détection précoce et son blocage approprié pourraient avoir un impact sur le pronostic rénal. Le premier objectif de mon projet de doctorat était d'étudier la présence de cellules immunitaires sénescentes au cours de l'insuffisance rénale aiguë, grâce à une cartographie du système immunitaire des cellules immunitaires rénales chez de jeunes souris en condition d'IRA. Le séquençage de l'ARN à l'échelle de la cellule isolée (sc RNASeq) a été utilisé dans le modèle RM-IRA murin induit par le glycérol. Nous avons pu obtenir les caractéristiques transcriptomiques des cellules vivantes CD45⁺ triées à partir des reins 2 jours après la lésion. Une combinaison de sénolytiques (dasatinib/quercétine) a été administrée à des souris exposées à la RM-IRA. Le clustering non supervisé de près de 17 000 transcriptomes de cellules uniques a permis d'identifier 7 groupes de cellules immunitaires connus. Le sous clustering des cellules phagocytaires mononucléaires (MPC), y compris les monocytes, les macrophages et les cellules dendritiques, a révélé 9 sous-populations cellulaires distinctes modifiées différemment par la RM-IRA. Un groupe de macrophages était particulièrement intéressant car il se comportait comme un nœud critique dans la trajectoire reliant un groupe de cellules MCHII^{high} uniquement présentes dans la condition contrôle, à 2 autres groupes MCHII^{low} uniquement présents dans RM-AKI. Étant donné que ce cluster spécial exprimait des gènes caractéristiques de la sénescence, l'effet d'un traitement sénolytique combiné dasatinib/quercétine (DQ) a été évalué. Le traitement par DQ dans la RM-IRA a amélioré la fonction rénale et bloqué le changement phénotypique de F4/80^{high}CD11b^{low} à F4/80^{low}CD11b^{high} MPC. Cette approche a permis d'identifier de nouveaux sous-types myéloïdes rénaux après RM-IRA et a démasqué une population transitoire de macrophages affectée par les processus de sénescence cellulaire. Ce travail apporte la preuve de concept que l'immunosénescence se produit pendant l'IRA et que les sénolytiques sont des médicaments néphroprotecteurs potentiels. Le deuxième objectif de mon projet de doctorat était de décrire

l'effet du vieillissement sur la réponse à la RM-IRA, car les études comparant l'effet des différences de sexe et de l'âge dans le RM-AKI manquaient. Des souris mâles et femelles de deux groupes d'âge différents (8 semaines et 26 mois) ont été comparées après une RM sur une période de 37 jours. Des études préliminaires ont mis en évidence la sensibilité accrue des femmes au RM-AKI avec l'âge, soulignant un dimorphisme sexuel probable. L'augmentation des marqueurs rénaux et des marqueurs d'inflammation ainsi qu'une fibrose plus importante et des lésions de la structure rénale ont été observées chez les femmes plus âgées, ce qui corrobore notre hypothèse d'une sensibilité accrue des femmes au RM-AKI en fonction de l'âge. Ce travail de thèse met en lumière les processus d'immunosénescence au cours de l'IRA, le rôle du vieillissement dans la progression de l'IRA et l'utilisation prometteuse des sénolytiques.

Abstract

Acute kidney injury (AKI) is a frequent and severe pathological condition, associated with a risk of developing chronic kidney disease (CKD). While AKI incidence and morbi-mortality are significantly higher in the elderly than in younger patients, the high susceptibility of the aged population to AKI remains to be fully explained. Immunosenescence (IS) (so-called “ageing-induced changes in the immune system”) may be the common denominator between AKI, CKD, and Aging. Whereas it is acknowledged that IS can occur during ageing and CKD, we made the innovative hypothesis that IS could also occur during AKI and that its early detection and appropriate blockade could impact on renal prognosis. Because we first described the role of macrophage subtypes in rhabdomyolysis-induced AKI (RM-AKI), we wish to further explore the impact of immune cells in AKI. The first objective of my PhD project was to investigate the presence of senescent immune cells during AKI, thanks to the immune landscape of renal immune cells in young mice submitted to RM-AKI. Single-cell RNA sequencing was used in the murine glycerol-induced RM-AKI model to dissect the transcriptomic characteristics of CD45⁺ live cells sorted from kidneys 2 days after injury. A combination of senolytics (dasatinib/quermetin) was administered to mice exposed to RM-AKI. Unsupervised clustering of nearly 17,000 single-cell transcriptomes identified 7 known immune cell clusters. Sub-clustering of the mononuclear phagocyte cells (MPC), including monocytes, macrophages, and dendritic cells, revealed 9 distinct cell sub-populations differently modified by RM. One macrophage cluster was particularly interesting since it behaved as a critical node in a trajectory connecting one MHCII^{high} cluster only present in control to 2 MHCII^{low} clusters only present in RM-AKI. Because this crucial cluster expressed senescence hallmark genes, the effect of combined dasatinib/quermetin (DQ) senolytics treatment was evaluated. DQ treatment in RM-AKI improved kidney function and blocked the phenotypic switch from F4/80^{high}CD11b^{low} to F4/80^{low}CD11b^{high} MPC. scRNA-seq identified novel renal myeloid subtypes after RM-AKI and unmasked a transitional macrophage population affected by cellular senescence processes. This work provides proof of concept that immunosenescence occurs during AKI and that senolytics deserve attention as potential nephroprotective drugs. The second objective of my PhD project was to describe the effect of ageing on response to RM-AKI as studies comparing the effect of gender differences and age in RM-AKI were lacking. Male and female mice from two different age groups (8 weeks and 26 months) were compared after RM-AKI over a period of 37 days. Preliminary studies showcased the increased sensitivity of females to RM-AKI with age highlighting a probable sexual dimorphism. Upregulation of kidney and inflammation

markers and more prominent fibrosis and kidney structure damage were observed in older females correlating with our hypothesis of increased sensitivity of females to RM-AKI with respect to age. Further studies are needed to precise biological pathways implicated in sex and age observed differences. This thesis work sheds light on immunosenescence processes during AKI, the role of ageing in RM-AKI progression and the promising use of senolytics.

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List of Abbreviations

AKI	Acute Kidney Injury
KDIGO	Kidney Diseases: Improving Global Outcomes
CKD	Chronic Kidney Disease
GFR	Glomerular Filtration Rate
CK	Creatinine Kinase
RM	Rhabdomyolysis
RM-AKI	Rhabdomyolysis induced AKI
DC	Dendritic Cell
NK	Natural Killer
NKT	Natural Killer T Cells
DAMPs	Danger-associated pattern molecules
ECM	Extracellular matrix
TLR	Toll like receptor
MPCs	Mononuclear Phagocytic Cells
GFR	Glomerular Filtration Rate
TEC	Tubular epithelial cell
SASP	Senescence-associated secretory phenotype
DQ	Dasatinib Quercetin
scRNASeq	Single Cell RNA Sequencing
MHCII	Major Histocompatibility Complex II

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INTRODUCTION

Acute Kidney Injury

Acute Kidney Injury (AKI) has been defined by the KDIGO (Kidney Diseases: Improving Global Outcomes) clinical practices guidelines workgroup [1] as an abrupt decrease in kidney function characterized by an increase in serum creatinine (>0.3 mg/dL within 48 hours or ≥ 1.5 times baseline) or urine volume <0.5 mL/kg/h for 6 hours and is associated with a rapid diminution in the kidney excretory function causing an accumulation of urea, creatinine and other toxic factors in the body [2]. Furthermore, AKI episodes are known to possibly lead to progression towards the development of chronic kidney disease (CKD) or end-stage renal disease (ESRD) leading to repercussions on mortality, morbidity, long-term health and increasing cost of care [3, 4]. Similarly, an episode of AKI has an impact on other short-term adverse outcomes such as immune dysfunction, fluid overload, electrolyte or acid-base derangement and bleeding complications which can also lead to a long-term adverse effect on survival [5].

AKI has been mainly associated with a reduction in the oxygen and nutrient delivery to the nephrons leading to an increase in requirement of energy demands [6]. A variety of different risk factors are known to be responsible for the above, the most common being ischemia where the reduction in blood flow leads to cellular injury causing organ dysfunction [7]. Exposure to different conditions such as cardiac surgery, exposure to nephrotoxic drugs, critical illness, burns, radiocontrast agents, sepsis, etc. are known to lead to AKI [8]. Risk factors associated with comorbidities such as cardiovascular disease, cancer and complex surgery are responsible for AKI in community, hospital and critical care settings [9,10].

AKI affects an overall of 13.3 million people a year out of which 85% of cases are from developing countries and is furthermore responsible for approximately 1.7 million deaths per year [11]. Epidemiological studies have shown that the elderly population has a higher incidence of AKI. A large-scale retrospective study revealed that 15% of AKI cases were in the 65-79 years age group and 22% of cases were observed in the >80 years old age group. Moreover, the elderly population with AKI had a mortality rate of 10.3% in the 65-70 years age group and 19.6% in the $>80\%$ years old age group. The incidence of patients requiring

dialysis and those without renal recovery was higher in elderly patients when compared with younger patients [12]. Thus, ageing is a risk factor for AKI.

Chronic Kidney Disease

Persistent alterations in kidney structure or function is associated with a syndrome called Chronic Kidney Disease (CKD) which has major implications for the health of the individual [13, 14]. The KDIGO classification describes an individual with CKD as one who has abnormalities in kidney structure or function that persist for more than 3 months [13]. There are a multitude of risk factors responsible for the onset of CKD including congenital abnormalities, diabetes mellitus, monogenic kidney disease, poorly controlled arterial hypertension, prolonged exposure to nephrotoxins and previous episodes of AKI [15]. Risk factors such as obesity or diabetes mellitus are responsible for an increase in the number of CKD patients, with an incidence of approximately 843.6 million individuals worldwide in 2017 [16]. The Global Burden of Disease studies have shown CKD as a leading cause of worldwide mortality [17, 18].

There have been important investigations demonstrating that AKI and CKD are linked and possibly promote one other. CKD has been recognized as a risk factor for AKI due to a decrease in glomerular filtration rate (GFR) and an increase in proteinuria induced by AKI [19]. A variety of different studies have shown that AKI initiates or accelerates the progression towards CKD [20, 21, 22, 23]. This has extensively been studied in animal models where maladaptive kidney repair after AKI (vascular rarefaction, glomerulosclerosis, tubular loss, chronic intestinal inflammation and fibrosis) has been known to cause a state that mimics the accelerated functional decline of the kidney observed with ageing [24, 25, 26, 27].

Rhabdomyolysis induced Acute Kidney Injury

Among the different causes of AKI, RM-AKI is not very studied but accounts for 10% of AKI. However, it can be life-threatening, and its pathophysiology is not fully understood.

The rapid breakdown of damaged skeletal muscle is responsible for rhabdomyolysis (RM). A disruption of the skeletal muscle leads to destruction in the sarcolemma and a reduction in the muscle membrane to cytoskeleton connection. Skeletal muscle damage leads

to the release of intracellular muscle components such as creatinine kinase (CK), myoglobin, lactate dehydrogenase, aldolase, or electrolytes into the circulatory system. Mouse models of RM-AKI are generated through intramuscular injection of 50% glycerol in the hind legs. RM can be responsible for asymptomatic illnesses with moderate CK elevation, as well as more extreme CK elevations that can be life-threatening, with disseminated intravascular coagulation, electrolyte imbalances and AKI [28, 29]. Furthermore, the destruction in the cytoskeleton connection is responsible for myofiber fragility and increasing sensitivity to damage [30]. RM has three main clinical symptoms: myalgia, weakness and myoglobinuria. However, these symptoms can be misleading because the trinity has only been reported in <10% of patients whereas >50% of patients have no complaints of muscle pain or weakness [31]. One of the most serious complications of RM is AKI which has been reported to develop in 33% of patients [32]. Certain populations at risk such as post-operative patients, chronic users of lipid-lowering drugs, and morbidly obese patients have a high incidence of RM. Crush syndrome, which describes the destruction of muscle after trauma, compression or injury usually due to man-made and natural disasters is responsible for majority of the RM cases [33]. A multitude of different causes is also responsible for RM in adults such as heat stroke, seizure, strenuous exercise, seizure, hypokalaemia, delirium tremens, enzyme deficiencies, hypophosphatemia, burns, heat stroke myopathy, trauma, immobility, hypernatremia, hyponatremia, endocrinopathies, prolonged immobilization, arterial emboli, shock infections and medications [34, 35, 36, 37, 38]. Fall risk is one of the most frequent causes of RM in the elderly populations with approximately 34% of the elderly in the age group of 65 years and above having at least one fall per year [39]. Reports have showcased the huge relationship between RM and ageing where 7-10% of AKI cases due to RM are observed in adults [40] while a significant increase to 68.9% was observed in the elderly [41].

Inflammation in Acute Kidney Injury

Inflammation is an elaborate immune response that occurs after an injury or infection and that leads to tissue repair and recovery of tissue homeostasis. Inflammation must be controlled otherwise it can further lead to autoimmune disorders, tissue damage and fibrosis [42].

Inflammation is now considered crucial in AKI-associated kidney disorders. Two different aspects of the immune system are responsible for the resulting inflammation response: innate

and adaptive immunity. Activation of the innate immune system occurs immediately after the infection or inflammatory stage and is non-antigen specific. The cells responsible for the innate immune system response are the following: dendritic cells (DCs), neutrophils, macrophages, monocytes, natural killer (NK) cells and natural killer T (NKT) cells. The adaptive immune system is activated only in response to certain antigens over a few days. The factors responsible for adaptive immune response are as follows: CD4 and CD8 T lymphocyte proliferation and activation, DC maturation and antigen presentation and T-to-B lymphocyte interactions (Figure 1). Macrophages and DCs are active components in both innate and adaptive immune responses and have an important function in the production of pro-inflammatory cytokines and the presentation of antigens to lymphocytes [43]. An early-stage inflammatory response corresponds to an innate immune response where the release of cytokines leads to the recruitment of macrophages and neutrophils at the site of injury. In the later stages of the AKI, adaptive immunity is taking place. Nevertheless, a recent study indicates the participation of T cells in early inflammatory response due to AKI [44].

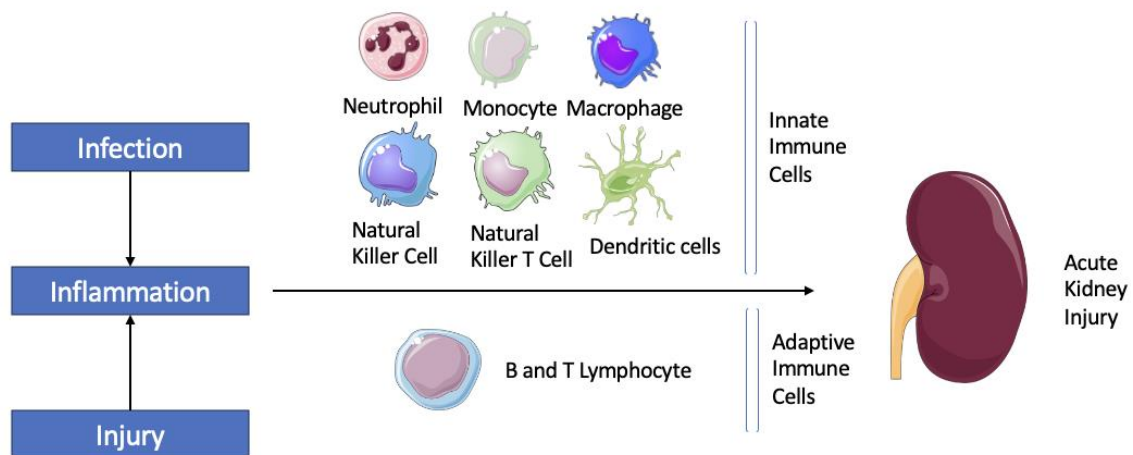


Figure 1: Factors responsible for the initiation of an inflammatory response leading to AKI
Adapted from [42, 43]

Damage-associated molecular patterns (DAMPs) are molecules that are released under injury. Passive release of DAMPs can be produced by dying cells or by disruption of plasma membrane and/or extracellular matrix (ECM) damage [45]. During sterile inflammation, innate immune cells respond to DAMPs instead of foreign-pathogen-associated molecular pattern molecules. DAMPs recognition occurs through Toll-like receptors (TLRs) which act as pattern

recognition receptors. These receptors contain three main components: an extracellular domain responsible for interaction with the endogenous DAMPs, a transmembrane domain and finally an intracellular signalling domain [46]. In the kidney, reactive oxygen species production leads to tubular cell death and DAMPs release which in turn control immune system modulators including mitochondrial material (adenosine triphosphate or high mobility group box one (HMGB1)), ECM proteins (fibronectin, hyaluronic acid, heparin sulphate) or cytoplasmic material (heat shock proteins (HSPs), uric acid). Studies have shown the importance of HSPs and HMGB1 in AKI [47]. Overall, DAMPs play an important role in the stimulation and amplification of an inflammatory response and tissue damage acceleration [48].

AKI has been known to be a disease affected by innate and adaptive immunity which further affects renal injury and up to recovery from AKI. Monocytes/macrophages, neutrophils, T cells, B cells and dendritic cells all play a role in AKI development. Specifically, regulatory T cells and M2 macrophages have been known to affect inflammation and AKI repair. Cytokines and neutrophils have a pivotal role in immune dysfunction particularly in bacterial clearance after AKI [49,50].

Inflammation and immune response in RM-AKI

The toxicity of myoglobin released by damaged muscle is not the only reason for RM-AKI induction. Other products are also responsible such as the immunogenic DAMPs that activate resident macrophages and favour the recruitment of circulating immune cells into the kidney interstitium. The production of inflammatory cytokines and cytotoxic molecules by immune cells is responsible for cell damage and the generation of a positive feedback loop. The immune system has a very important role in RM-AKI but the complete role of the immune system in RM-AKI has not been fully studied.

Complement deposits have been observed in mice and patient kidneys with RM-AKI. The complement system has been known to be a bridge between innate and adaptive immune response [51], complement system activation was shown to be an important factor in RM-AKI where the complement component 3 (C3) was shown to be responsible for the activation of the complement system. The alternative and lectin pathways both have a role in the complement activation in the kidney due to RM-AKI [52].

The primary immune system response of RM-AKI is innate immune response while the secondary response of RM-AKI is due to adaptive immune response which takes at least 1 week to occur. The molecular mechanism of RM-AKI is as follows (Figure 2):

1. There is an infiltration in myoglobin into the interstitium which has a predominant role in immune activation.
2. Myoglobin further leads to the downstream activation of tubular cells which secrete macrophage chemoattractants like *Ccl2* and *Ccl7*.
3. The chemoattractants then lead to the migration of blood monocytes into the renal interstitium.
4. The blood monocytes released are further affected through myoglobin which causes macrophage polarization towards a proinflammatory phenotype.
5. $CD11b^{high}F4/80^{low}Ly6B^{high}$ macrophages lead to renal injury augmentation by secretion of proinflammatory cytokines and ECM compounds.

Innate immune cells such as macrophages, monocytes, neutrophils, and natural killer cells are the preliminary immune cells affecting RM-AKI [50, 53].

The role of immune cells particularly macrophages in RM-AKI development has been described but the detailed changes in the immune cells during RM-AKI have not been established.

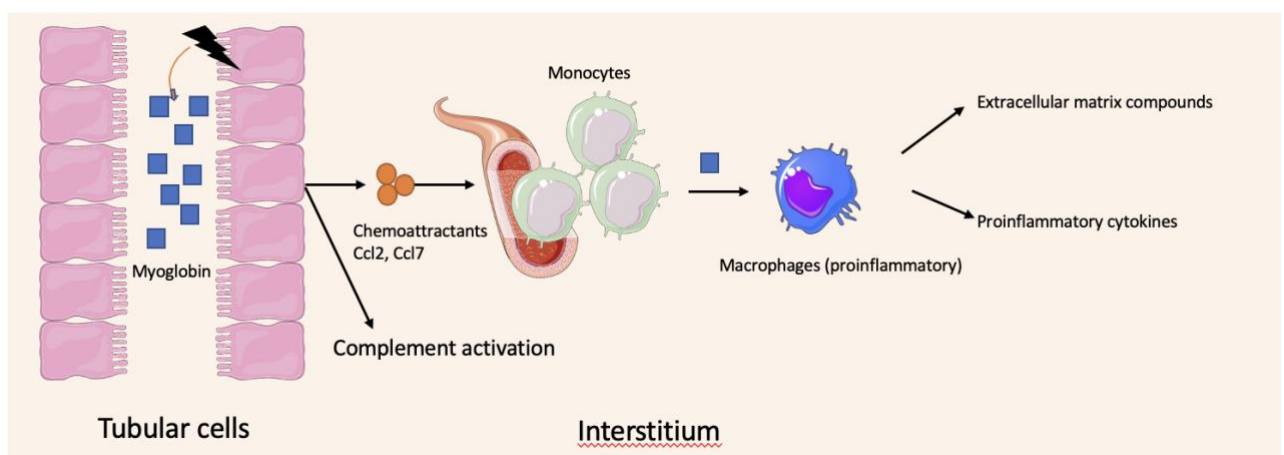


Figure 2: Mechanism of kidney inflammation during RM-AKI

Adapted from [50, 52, 53]

Mononuclear Phagocytic Cells in AKI

Mononuclear Phagocytes (MPCs) include macrophages, monocytes, and dendritic cells (DCs) [54,55]. Monocytes are developed from the bone marrow and are present in the circulatory system and further travel to the peripheral organs. Monocytes differentiate into macrophages and are responsible for tissue homeostasis through growth factor production and apoptotic body phagocytosis. DCs have a role in antigen presentation to T lymphocytes. DCs also have an important role in processing antigens. Monocytes, some macrophage subsets, and the majority of DCs originate from hematopoietic stem cell-derived progenitors [55,56]. Inflammation is responsible for an increase in circulatory monocytes towards the targeted organs and leading to conversion into inflammatory MPCs with DC and macrophage characteristics [55].

Macrophage polarization occurs through microenvironmental stimuli leading to distinct functional phenotypes of macrophages. There are classically activated (M1) and alternatively activated (M2) macrophages after macrophage polarization. The two macrophage subtypes differ in their secreted cytokines, biological functions, and cell surface markers. The pathways responsible for the polarization are due to arginine metabolism. M1-like macrophages are reported to be arising as products from the iNOS pathway while M2-like macrophages are by-products of the arginase pathway [57]. It is important to note that the M1/M2 classification has been reported to be oversimplified due to the heterogeneous nature of M1/M2 macrophages and it could be more apt to state that macrophages are M1 or M2-like rather than stating the conventional nomenclature [58].

Some studies on rodent models have shown the importance of macrophages in RM-AKI. In RM-AKI, macrophages from circulating monocytes migrate from kidney resident cells or the tissue [50].

Three populations of MPCs were identified after RM-AKI induction in mice and they were as follows: F4/80⁺CD11b⁺ (R0), F4/80^{low}CD11b^{high} (R1) and F4/80^{high}CD11b⁺ (R2) [53]. The R1 macrophage subset was observed to increase in the RM-AKI condition two days after glycerol injection for induction of RM in mice. The R1 macrophages were observed to express a high level of *Ccl2* and *Ccl7* receptors. The R1 macrophages migrate from the blood to the kidney interstitium and a high expression of Ly6b indicates that R1 macrophages originate from the

monocytes. R1-Gly (R1 macrophages after glycerol injection) revealed a differentiation pattern towards M2 while R2-Gly (R2 macrophages after glycerol injection) was reported to differentiate into M2b macrophages. Eight to thirty days after glycerol injection, the return towards the R2 dominant phase was detected implying a reversion from the proinflammatory towards a repairing stage [53,59]. The R2 macrophages might have a possible role in renal repair and are further associated with extracellular matrix degradation due to an increase in enzyme expression by *MMP-13*. Depletion of macrophages through clodronate ascertained the role of macrophages in RM-AKI [53,60]. Another study showed the presence of CD163-positive macrophages in kidney biopsy from RM-AKI patients, with a M1 response in the early stage due to myoglobin and a partial polarization towards M2 in later stages. These myoglobin-derived macrophages were then hypothesized to progress towards fibrosis through the production of CTGF and TGF- β (fibrosis markers) (Figure 3) [61].

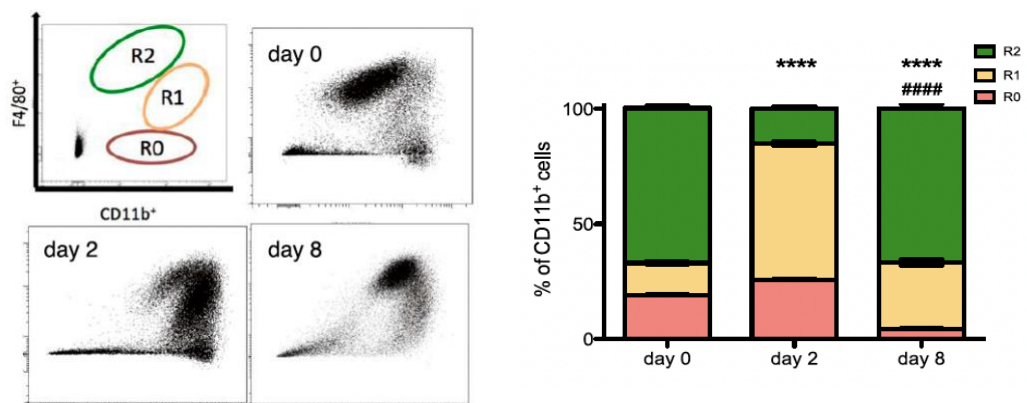


Figure 3: Reversal of MPC population observed over time in RM-AKI

From [53]

The role of macrophages in RM-AKI development has been described and some of the macrophage subtypes have been identified through traditional flow cytometry and basic single-cell sequencing with a limited gene set. There is a need to perform high throughput sequencing to understand the MPC heterogeneity and the MPC subtypes. These subsets may give information on the progression of RM-AKI and can further be used to delve into the different possible pharmacological treatment strategies.

Renal Ageing

Age is associated with a progressive loss of functional reserve of the organs and a decline in the homeostatic capacity resist to internal and/or external stress, and this results in an increasing risk of diseases and death [62]. Renal ageing results from a multitude of different environmental changes, genetics, and cellular dysfunction [63].

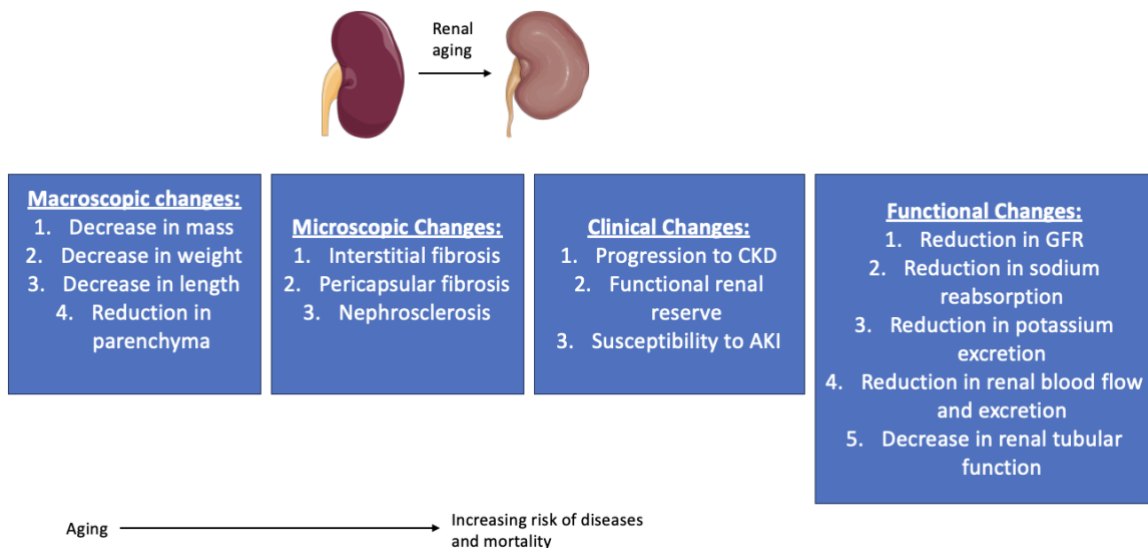


Figure 4: Structural and functional changes in aging kidney

Adapted from [64]

The different changes that characterize renal ageing are as follows (shown in Figures 4 and 5):

- A decline in the nephron size and number, glomerular basement membrane thickening, tubulointerstitial alterations and increased glomerulosclerosis.
- In longitudinal studies, a decline in the Glomerular Filtration Rate (GFR) is observed with age.
- Increased susceptibility to AKI due to reduced sodium absorption, potassium excretion, progressive tubular dysfunction, and reduction in urine concentration capacity.
- A multitude of vascular alterations such as increased extracellular mass deposition, increased intrarenal shunting, increased cell proliferation in preglomerular arterioles and reduced efficacy of renal vasodilators such as nitric oxide (NO).
- A reduction in Klotho (a transmembrane protein which is a coreceptor for fibroblast growth factor [FGF] that is strongly expressed in the kidney) which is involved in

hypoxia, cellular regeneration, and senescence. Antifibrotic effects of Klotho are further responsible for the modulation of Wnt signalling.

- A decrease in nuclear receptor Peroxisome Proliferator-Activated Receptor gamma (PPAR γ), the activation of which increases Klotho expression.
- An increase in angiotensin II was observed in older rats when compared with younger control rats and led to glomerular cell growth and increased fibrosis/ECM accumulation.
- Oxidative stress has been known to contribute to the ageing phenotype [64].

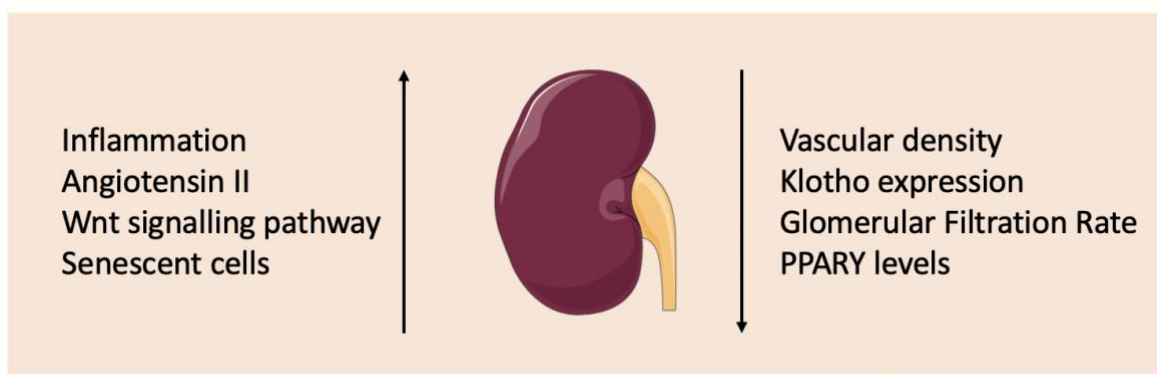


Figure 5: Factors altered due to renal ageing
Adapted from [64]

Recent studies have showcased senescent cells to be increased in diseases such as chronic nephropathy and has furthermore been shown to progress towards fibrosis. Multitudes of studies have showcased the association of senescent cells in aged and disease-affected human renal biopsies. These studies have brought to light the association of cellular senescence as a process involved in the early and late phases of renal ageing with a connection to fibrosis [64,65].

The role of renal ageing has been described in AKI and the association of senescent cells have been explained in the case of CKD. There is a need to perform studies analyzing the effect of ageing on RM-AKI. Similarly, it is of essence to describe the underlying effect of senescent cells in RM-AKI in the disease condition and whether senescent cells can further progress the disease from RM-AKI to CKD condition.

Cellular Senescence

Cellular senescence has been defined by cell cycle arrest that is permanent and has been explained to have an anti-proliferative response with a prominent role in ageing and injury. Cell cycle arrest can occur due to a variety of cellular stresses. Senescence occurs due to a multitude of different factors such as telomere shortening and repeated cell division. With age, an increased number of senescent cells accumulate in the organs which are usually identified by factors such as p16^{INK4a} or senescence-associated beta-galactosidase expression which are common biomarkers. A variety of different cytokines and growth factors have been showcased to have a role in the induction of senescence. Senescent cells can act autonomously causing induction of cell cycle arrest and simultaneously non-autonomously through the help of proteins associated with neighbouring cells known as senescence-associated secretory phenotype (SASP) [64,65] (Figure 6). Senescent cells are observed to be more resistant to apoptosis [66].

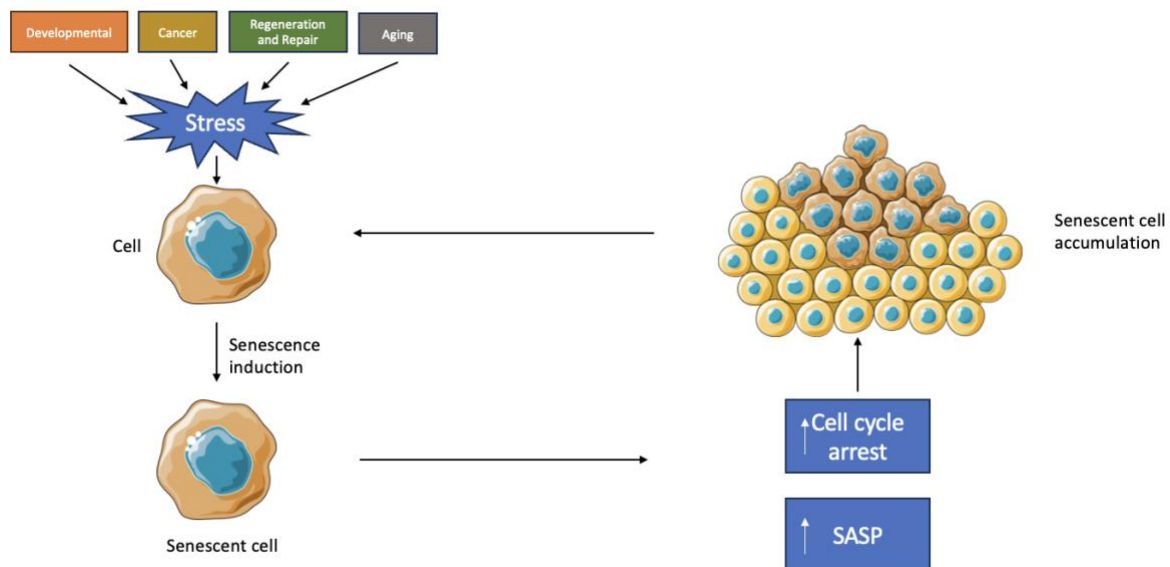


Figure 6: Factors triggering cellular senescence

Adapted from [65]

There are a variety of different signalling pathways that are associated with renal ageing and cellular senescence, but the most predominant ones are that of the p53/p21 and p16/Rb pathways. The pathways are usually activated due to DNA damage caused by a multitude of

stresses. The activation of p21^{CIP1} and p16^{INK4a} leads to suppression of CDK complexes and retinoblastoma (Rb) protein. Renal cell senescence and kidney ageing furthermore occur due to the halting of cell proliferation by Rb. This downstream leads to impairment of reparation and exposes the kidney to a variety of different injuries. Klotho which has a major role in cell aging modulates cellular senescence. Different signalling molecules have been identified that have a role in cellular senescence and Wnt9a/beta-catenin signalling has been reported to play a role in the promotion of renal fibrosis and renal tubular senescence in diseased kidneys where upregulation of expression of p16^{INK4a}, p21 and p53 as well as an increased activity of SA-beta-gal in renal tubules [66,67]. Acute senescent and chronic senescent are the two main types of senescent cells where acute senescent cells are present transiently while chronic senescent cells are developed through prolonged signalling and senescent cell accumulation. Age-related decline of kidney function has further been associated with chronic senescent cells which accumulate in the kidney due to natural aging. Senescent cells have been associated with a multitude of renal diseases and are associated with disease progression and deterioration [67] (Figure 7).

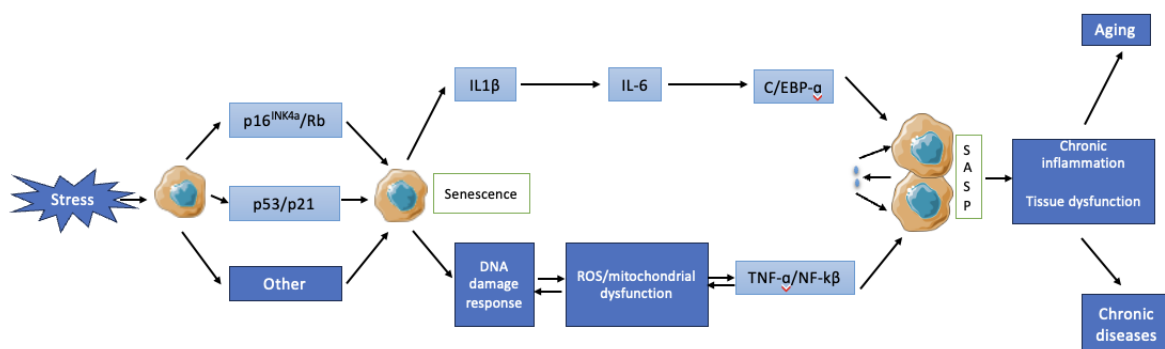


Figure 7: Pathways responsible for senescent cell induction

Adapted from [67]

In AKI, the regions that undergo senescence are the cortex and medulla but along with these podocytes, renal tubule epithelial cells (TECs), endothelial cells, mesenchymal cells, and vascular smooth muscle cells also undergo senescence. Tubular cell senescence has been known to be a central mechanism leading to the accumulation of senescent cells after injury and leading towards the progression of kidney damage. SASP has also been shown to have a high presence in the acute phases of AKI and CKD [68]. SASP is increased in elderly mice

with AKI [69] and SASP secretion has been shown to further aggravate cell senescence and fibrosis leading to CKD [70].

Compounds that allow the elimination of senescent cells could be a probable therapeutic mechanism for age-related pathologies and kidney disease-related pathologies.

Seno-therapeutics and Senolytics

Seno-therapeutics are compounds that eliminate senescent cells. It is a growing agent as a therapeutic target for the treatment of a variety of disorders and diseases associated with senescent cell accumulation from neurodegenerative disease, cancers, atherosclerosis, diabetes, kidney injury, etc. [71]. As senescent cell viability is dependent on apoptosis resistance and anti-apoptotic signalling, therapeutic strategies that have been developed for oncology can be modified for the treatment of complications due to kidney ageing. Seno-therapeutic drugs include different types of molecules such as senolytics which are compounds that selectively kill senescent cells, senomorphics which are compounds that inhibit SASP, modulate morphology and function of senescent cells and/or delay progression towards senescent cells and finally senoinflammatory mediators which are immune-system effectors which are responsible for the clearance of senescent cells [72] (Figure 8).

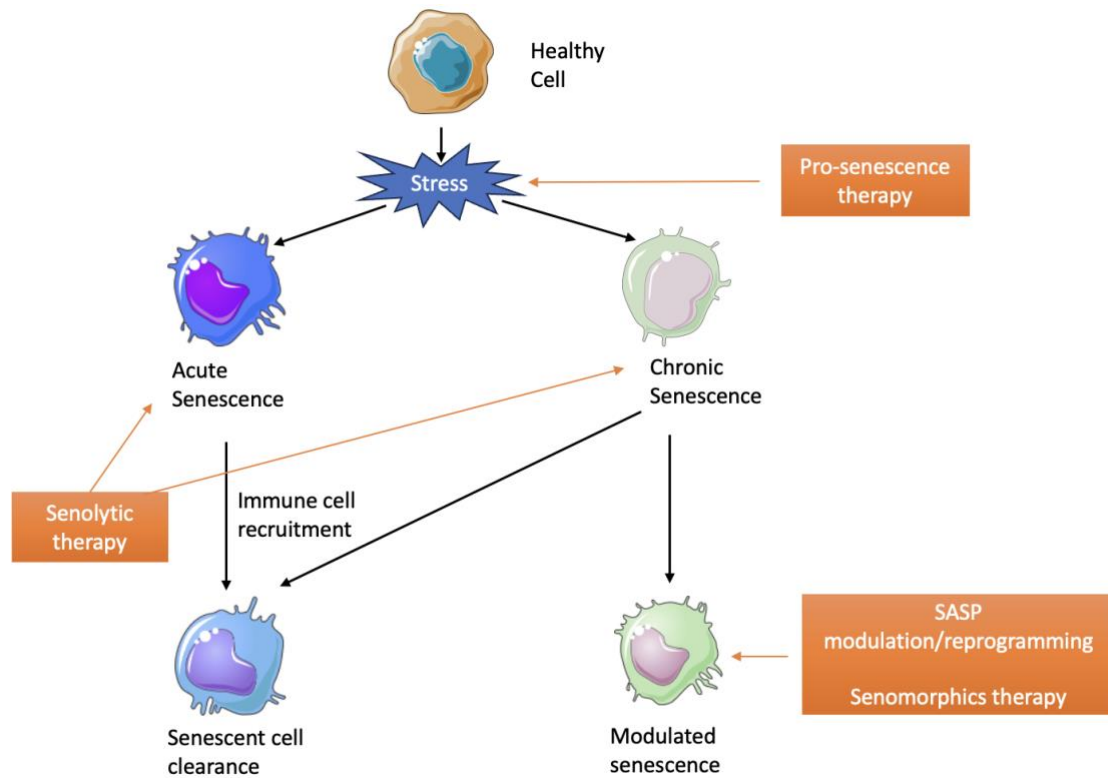


Figure 8: Senotherapeutics methodology of action

Adapted from [67, 72]

Senolytics have been identified as a method for the treatment of age-related diseases and could further promote healthy ageing, this was identified through studies of senescent cell depletion in INK-ATTAC transgenic mice [73, 74]. Senolysis treatment can then be applied based on the same strategy for the treatment of cancer cells which involves activation of the immune system, activation of pro-apoptotic pathways and activation of pro-survival pathways [67]. Senolytic drugs that specifically target senescent cell apoptosis and do not affect non-senescent cells are predominantly used. Some of the senolytics identified as the first generation are navitoclax, dasatinib and quercetin. Dasatinib is a tyrosine kinase inhibitor which acts through suppression of apoptosis through inhibition of ephrin B. It is a FDA-approved drug for the treatment of chronic myeloid leukaemia (NDA #0211986 and 022072). Cell death is caused by quercetin through inhibition of phosphoinositide 3 kinase [75]. The senolytics effect of dasatinib and quercetin have been widely studied in a variety of cell lines as well as mouse models for diseases including Alzheimer's, fibrotic pulmonary disease and age-dependent hepatic steatosis [76, 77, 78]. Studies have showcased how dasatinib and quercetin treatment have reduced senescent cell burden in multiple cisplatin treatment murine models and renal unilateral ischemia-reperfusion injury mice models. Post ischemic AKI, a reduction in kidney

fibrosis was observed as well. A recent pilot clinical study where a combination treatment of dasatinib and quercetin was used for the treatment of patients with diabetic chronic kidney disease showcased a reduction in senescent burden in skin biopsy specimens and adipose tissue after just 11 days of treatment [79].

The possibility of the use of senolytics for the treatment of AKI and RM-AKI is an important area to be looked at since the current treatment strategies for RM-AKI have their limitations as predominantly supportive care is used which requires prolonged access to medical professionals, supplies and advanced modalities such as dialysis.

Immunosenescence and Inflammaging

Aging accounts for changes in the immune system in both the function and the composition which leads to a deterioration in the immune system (both innate and adaptive) which is known as immunosenescence. Immunosenescence leads to a reduction in response to vaccinations, increased incidence of autoimmune disorders, increased susceptibility to infections, systemic inflammation, and maladaptive tissue repair. Immunosenescence affects both innate and adaptive immunity, where the defective immune response accounts for thymic involution, hematopoietic stem cells deterioration, accumulation of immune and non-immune senescent cells, induction of pro-inflammatory immunological memory and a signature of pro-inflammatory signals (inflammaging). Immunosenescence usually affects humans above the age of 65 [80].

The immune cell populations are altered due to immunosenescence. A reduction in the following immune cell subpopulations occurs:

1. Natural killer cells that are responsible for the recognition and clearance of tumour cells are viral infections.
2. Macrophages are responsible for antigen processing and presentation.
3. Antigen-presenting cells.
4. Naïve T cells that are produced from the thymus.
5. T-cell response due to decreased antigen stimulation by antigen-presenting cells.
6. Humoral immunity is impaired due to the fewer B cell stimulation.

An increase in memory B and T cells takes place [81] (Figure 9).

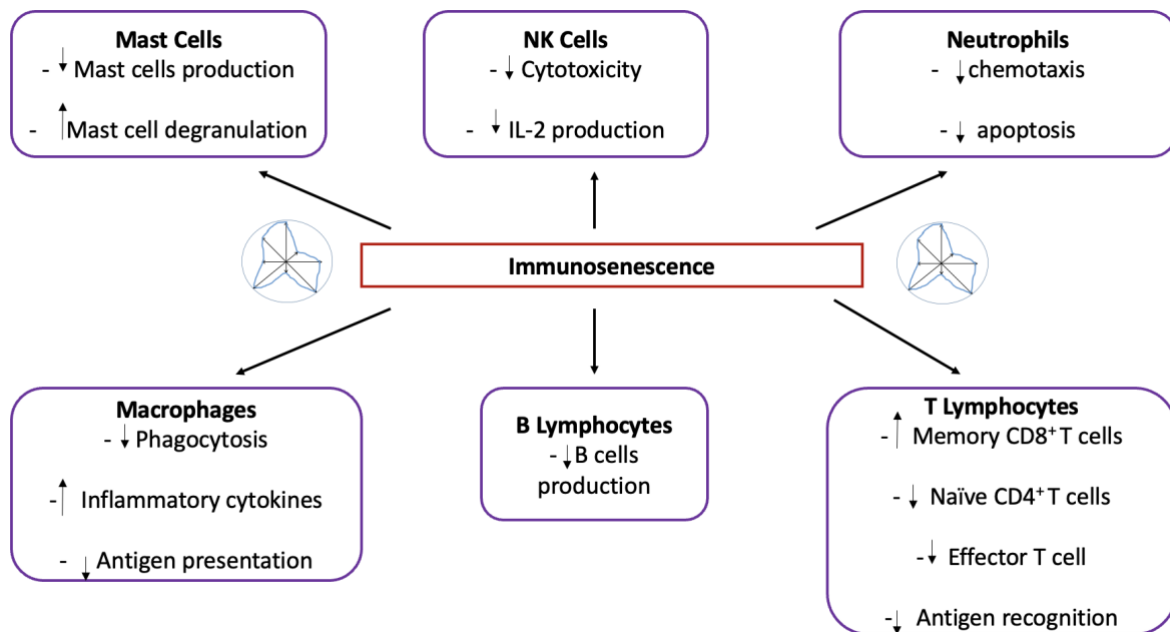


Figure 9: Immune cell populations altered due to immunosenescence

Adapted from [82, 83]

When the immunological age and chronological age of patients with CKD, ESRD, autoimmune disease and chronic infections were compared with that of an individual undergoing healthy ageing it was observed that patients with CKD, etc. had an accelerated immunological age with a difference of 20 years when compared with an age-matched healthy individual. Similarly, the immunological characteristics of a patient with CKD were compared with an elderly individual with immunosenescence and some similarities were observed:

1. CD4/CD8 ratio reduction.
2. Reduction in number of lymphocytes and an increase in myeloid cells.
3. Decrease in the phagocytic activity of macrophages and monocytes.
4. Increase in pro-inflammatory T cells and a reduction in naïve t cells.

On the other hand, as previously discussed aging leads to an increase in levels of inflammation leading to the promotion and progression of different kidney diseases. Inflammaging might also be responsible for the high incidence of AKI and the progression towards CKD in the elderly [80].

Due to the studies showcasing the similarities between an immunosenescent individual and a patient with AKI, it is of utmost importance to look at the association of immunosenescence in AKI and RM-AKI.

CONCLUSION

Ageing leads to a higher susceptibility to develop AKI and CKD. Ageing is related to a higher risk of RM-AKI. Because we already studied immune processes involved in RM-AKI prognosis on young individuals, we wished to explore whether AKI-mediated ageing processes occurred in young individuals and whether ageing could affect immune response during RM-AKI.

The objectives of my thesis are the following:

1. Does immunosenescence occur during RM-AKI?
2. Does ageing affect the response to RM-AKI?

PERSONAL WORK

Introduction

The incidence of AKI after an RM event reaches 80% of individuals. The impact of RM-AKI in the short and long term has been well studied. When it comes to RM-AKI treatment, there are no specific treatment options available and the role of different immune cells in the initiation and transition of the disease from control to RM-AKI condition needs further investigation.

It is well known that MPCs play an important role in RM-AKI and that their depletion can further reverse the phenotype. R1 macrophages (F4/80^{low}CD11b^{high}) were identified in RM-AKI through traditional flow cytometry where R1 macrophages were observed to increase 2 days after RM induction and showcased a probable inflammatory phenotype. This macrophage population diminished 8 days after RM induction which showed signs of a repair phase, in this stage R2 macrophages (F4/80^{high}CD11b⁺) were observed to be the majority. Clodronate which has been known to remove macrophages were tested for RM-AKI and the RM-AKI-associated macrophages were observed to be diminished, but this treatment strategy cannot be used in clinics due to its absence of specificity. Single-cell transcriptional analysis of R1 and R2 macrophages have been performed but the information obtained previously [53] was limited due to the limited gene set for the study. Due to the limitations in the previous studies, there was a need to dive deeper into the immune landscape in the RM-AKI condition and to zoom into the probable MPC subpopulations through single-cell RNA sequencing with a wider gene set. Furthermore, information obtained from high throughput studies can give an array of information on aspects of the immune system that could then be targeted for a highly specific treatment strategy.

Single-cell RNA sequencing (scRNASeq) analysis of CD45⁺ live kidney cells (from FACS sorting) 2 days after RM-AKI induction was performed. This led to the identification of 7 different immune cell clusters including macrophages, monocytes, neutrophils, T cells, B cells, DCs and NKs. A relative increase of macrophages, monocytes and neutrophils was observed in the RM-AKI condition over the control condition. As the MPCs were one of the most highly observed immune cells in the infiltrate, they were zoomed into, and the different subpopulations were observed. Certain clusters were observed exclusively in control and RM-

AKI conditions respectively. Cluster 8 was a peculiar cluster as it was observed in both conditions and seemed to increase in the RM-AKI condition. The MPC clusters obtained were compared with the previously known MPC showcasing that some of our clusters could be classified as R1 and R2 respectively.

As cluster 8 was observed to increase in RM-AKI condition, it was of particular interest to delve further into this subpopulation. Cluster 8 was observed to be at the junction between control and RM-AKI clusters in trajectory analysis. To further analyse the characteristics of cluster 8, the top differentially expressed genes were compared. It was observed that major markers of the major histocompatibility complex II (MHCII) were the top downregulated genes. Interestingly, the expression of these MHCII markers was higher in the control clusters while it was lower in the RM-AKI clusters. Indicating two classifications of MHCII^{high} and MHCII^{low} respectively. Cluster 8 meanwhile showcased a bimodal expression of the MHCII transcripts indicating the possibility that this cluster is acting as a transition cluster between the control and RM-AKI condition where there is reprogramming of the MHCII^{high} cells to MHCII^{low} cells.

To further analyse the characteristics of cluster 8, KEGG analysis was performed, and it was observed that enrichment of cell cycle and cellular senescence-associated genes were observed when compared with the other MPC clusters. Reactome enrichment analysis also showcased that the pathways of cell cycle and cellular senescence were predominant. These details and the association that these pathways have with those of cell cycle enrichment give the probable indication that this cluster could have a role in MPC renewal during injury.

To investigate the role of tubular epithelial cells (TECs) in cellular senescence, publicly available datasets were analysed since TECs are known to interact with infiltrating immune cells. The gene sets were compared with that of cluster 8 and none of the TEC-enriched genes were observed indicating that cellular senescence could vary based on the specific renal cell location.

Ageing markers p21 and Fgf2 were observed to be upregulated 2 days after RM-AKI induction giving another indication along with the fact that cellular senescence is a prominent pathway in cluster 8 that senolytics could be a possible treatment strategy. A combination treatment of DQ was performed where administration was done daily, 3 days before and 2 days after RM-

AKI induction through 50% glycerol injection. No modifications in CK were observed indicating the treatment did not affect RM. On the other hand, DQ treatment significantly reduced the BUN indicating a possible preventive role against RM-AKI. DQ treatment showcased a reversion of the MPC phenotypic switch, which was observed through traditional flow cytometry, which was also observed 7 days after RM-AKI induction. The kidney function was observed to significantly improve over time.

Overall, this study gives a deeper array of information on the immune cells and MPCs affecting RM-AKI. It gave light to a MPC subpopulation affected by cellular senescence processes. This work sheds light on the possibility of using senolytics as a treatment strategy for RM-AKI.

Manuscript

This work has been presented or published in the following:

- Poster presented with the title “Unravel Immunosenescence Role in Response to Kidney Injury: A First Step Immune Landscaping” at the 2nd EuroGeroscience Conference, 24-25 March 2022, Toulouse
- Mini Oral presented with the title “Immunolandscaping of Rhabdomyolysis-Induced Acute Kidney Injury” at the 59th Congress of the European Renal Association, 19-22 May 2022, Paris
- Oral presentation done with the title “A first step in immune landscaping in response to rhabdomyolysis induced acute kidney injury” at the doctoral school days program organized by the Biology, Health and Biotechnology Doctoral school in the University of Toulouse III Paul Sabatier, 29-30 September 2022
- Abstract published titled “Immune Landscaping in Rhabdomyolysis induced Acute Kidney Injury Identifies Senescence as a Potential Target” in the conference supplement of Nephrology Dialysis Transplantation for the 60th European Renal Association Congress

This work was accepted on 18/07/2023 for publication in the journal Nephrology Dialysis Transplantation (NDT).



Single-cell RNA sequencing identifies senescence as therapeutic target in rhabdomyolysis-induced acute kidney injury

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ABSTRACT

Background. The role of macrophages in the development of rhabdomyolysis-induced acute kidney injury (RM-AKI) has been established, but an in-depth understanding of the changes in the immune landscape could help to improve targeted strategies. Whereas senescence is usually associated with chronic kidney processes, we also wished to explore whether senescence could also occur in AKI and whether senolytics could act on immune cells.

Methods. Single-cell RNA sequencing was used in the murine glycerol-induced RM-AKI model to dissect the transcriptomic characteristics of CD45⁺ live cells sorted from kidneys 2 days after injury. Public datasets from murine AKI models were reanalysed to explore cellular senescence signature in tubular epithelial cells (TECs). A combination of senolytics (dasatinib and quercetin, DQ) was administered to mice exposed or not to RM-AKI.

Results. Unsupervised clustering of nearly 17 000 single-cell transcriptomes identified seven known immune cell clusters. Sub-clustering of the mononuclear phagocyte cells revealed nine distinct cell sub-populations differently modified with RM. One macrophage cluster was particularly interesting since it behaved as a critical node in a trajectory connecting one major histocompatibility complex class II^{high} (MHCII^{high}) cluster only present in Control to two MHCII^{low} clusters only present in RM-AKI. This critical cluster expressed a senescence gene signature, that was very different from that of the TECs. Senolytic DQ treatment blocked the switch from a F4/80^{high}CD11b^{low} to F4/80^{low}CD11b^{high} phenotype, which correlated with prolonged nephroprotection in RM-AKI.

Conclusions. Single-cell RNA sequencing unmasked novel transitional macrophage subpopulation associated with RM-AKI characterized by the activation of cellular senescence processes. This work provides a proof-of-concept that senolytics nephroprotective effects may rely, at least in part, on subtle immune modulation.

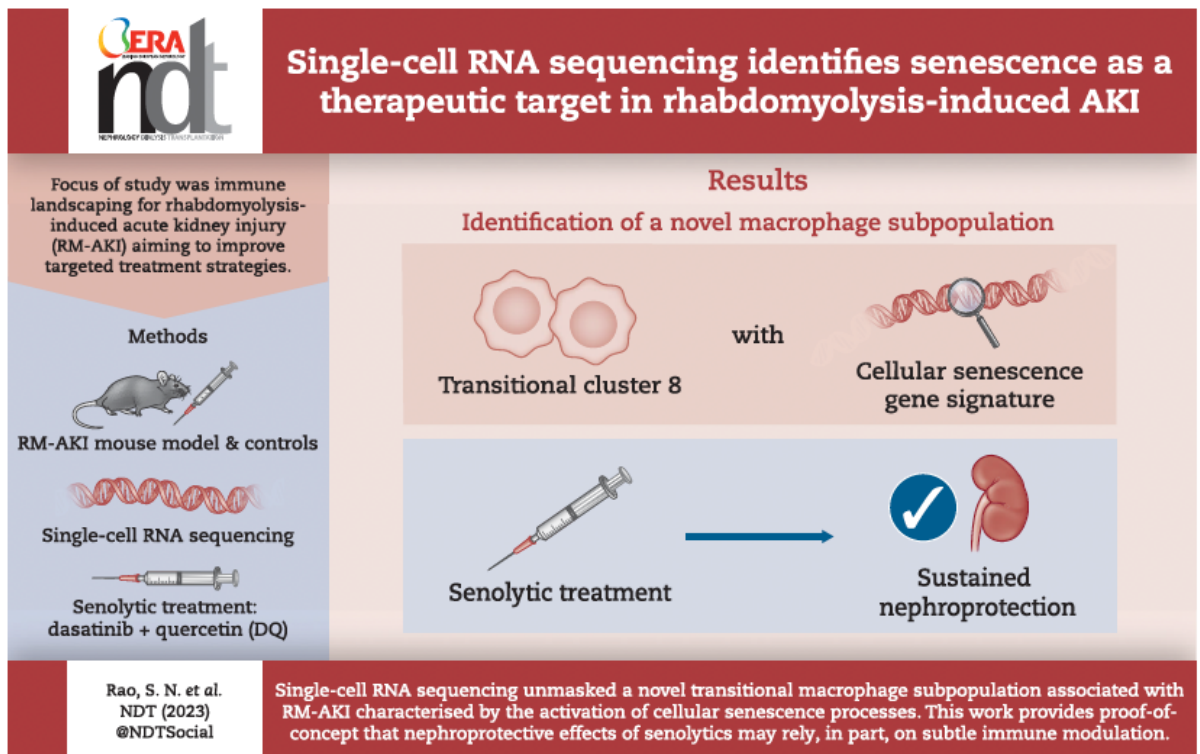
Keywords: AKI, mononuclear phagocytic cells, rhabdomyolysis, senescence, single-cell RNA sequencing

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GRAPHICAL ABSTRACT



KEY LEARNING POINTS

What was known:

- Specific treatment of rhabdomyolysis-induced acute kidney injury (RM-AKI) is lacking.
- Kidney macrophages play an important role in RM-AKI progression and repair.

This study adds:

- A high-resolution single-cell RNA sequencing-based atlas of the immune cells affected by RM-AKI in the kidney.
- The identification of a transitional macrophage subpopulation characterized by the activation of cellular senescence processes and involved in reprogramming of other macrophage subpopulations.
- The preventive effect of senolytics against RM-AKI.
- A proof-of-concept that senescence occurs during AKI both in immune and tubular epithelial cells (TECs).

Potential impact:

- Providing a proof-of-concept that senescence occurs during AKI in immune cells as well as TECs.
- Senolytics should deserve attention as potential nephroprotective drugs at early timepoints in AKI.

INTRODUCTION

Rhabdomyolysis (RM) frequently occurs in crush injury or after excessive drugs intake [1]. RM-induced acute kidney injury (RM-AKI) is reported in 80% of individuals experiencing an RM event, which has a serious impact on prognosis in the short and long term [2]. Specific RM-AKI treatment is lacking and the detailed mechanisms of the injury are unclear, especially with respect to the immune system. Depletion of mononuclear phagocytic cells (MPC), especially R1 macrophages (F4/80^{low}CD11b^{high}), improved

kidney repair and mouse survival, and reduced long-term fibrosis in a RM-AKI mouse model [3]. High-resolution immune cell profiling using single-cell RNA sequencing (scRNASeq) recently highlighted a role for regulatory T cells in kidney disease progression after ischaemia-reperfusion induced AKI [4], suggesting that obtaining high resolution information on both innate and adaptive immune systems in RM-AKI may help to develop specific treatment strategies. We therefore generated a detailed atlas of the immune cell population, more specifically MPC, in RM-AKI by

scrRNASeq. Whereas senescence is usually associated with chronic kidney disease, we wished to explore whether senescence could also occur at the early phase of AKI and whether senolytics could also act on immune cells.

MATERIALS AND METHODS

Details can be found in the [Supplementary data](#).

Animal models and treatments

RM-AKI was induced in 8 weeks old C57BL/6J mice as previously described [5]. In senolytic treatment experiments, mice received daily intraperitoneal injections of vehicle or combined dasatinib (D) and quercetin (Q) (respectively 10 and 50 mg/kg) before and after RM-AKI. Sacrifice was performed 2 days after RM-AKI induction.

Preparation of single-cell suspension for scrRNASeq

The kidneys were rinsed and digested as previously reported [3]. CD45 live cells were sorted using a BD Influx Cell Sorter (BD Biosciences). The single cell suspensions underwent barcoding and cDNA library preparation using a 10X Chromium Single Cell Instrument. Sequencing was performed on a Novaseq 6000 (Illumina) according to the manufacturer's instructions.

Literature-based analysis of public single-cell or single-nuclei RNASeq datasets

GSE180420, GSE171417 and GSE139107 data sets were screened for 'Cellular senescence' Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway (mmu04218) [6–8].

Statistical analyses

Statistical analyses for scrRNASeq data are detailed in [Supplementary data](#). Unpaired two-tailed Mann–Whitney test and analysis of variance (ANOVA) were performed with GraphPad Prism, and $P < .05$ was considered as statistically significant.

RESULTS

RM-AKI drastically modifies the mononuclear phagocytic cell landscape in kidney

scrRNASeq analysis of FACS-sorted CD45⁺ live kidney cells 2 days after RM-AKI (Fig. 1A and [Supplementary data](#), Fig. S1A and B) identified seven cell clusters including macrophages, monocytes, T cells, B cells, dendritic cells (DC), neutrophils and natural killer T cells (NKT) (Fig. 1B and [Supplementary data](#), Fig. S2). RM-AKI was associated with a relative increase in macrophage, monocyte and neutrophil populations (Fig. 1C). Adaptive immune populations (B and T cells) were present in both conditions, but no particular polarization at the early phase of the immune response was observed. Functional enrichment analyses are available in [Supplementary data](#), Fig. S3A and B.

Since the MPC were the most abundant immune cells within the infiltrate (Fig. 1C), we analysed this population in more detail. Nine major MPC sub-clusters were identified (Fig. 1D and E). Clusters 0–4, 6, 8 and 9 corresponded to macrophages, Cluster 5 to monocytes and Cluster 7 to DC. Macrophage Clusters 1, 2 and 6 were found almost exclusively in Control conditions, where they represented 89% of the MPC. Macrophage Clusters 0, 3 and 4 were found almost exclusively in RM-AKI, where they represented 78% of the MPC. Monocyte Cluster 5 was found almost exclusively in

RM-AKI, where it represented 14% of the MPC. The DC Cluster 7 represented 6.9% of the MPC in Control and was reduced to 0.8% in RM-AKI (Fig. 1E). Cluster 8 was the only cluster that increased in RM-AKI (5% MPC) compared with Control (0.2% MPC).

We previously demonstrated that RM-AKI leads to a shift from dominant of F4/80^{high}CD11b^{low} (R2) MPC to dominant F4/80^{low}CD11b^{high} (R1–R0) MPC [3] ([Supplementary data](#), Fig. S4A). scrRNASeq ([Supplementary data](#), Fig. S4B) and CITE-seq analysis ([Supplementary data](#), Fig. S4C) showed that *Adgre1* (encoding F4/80) predominated in Clusters 1 and 6, suggesting that they could be classified as R2 MPC, and that *Itgam* (encoding CD11b) predominated in Cluster 4 and 5, suggesting that it could be classified as R0–R1. Comparison of our scrRNASeq MPC data with scrRNASeq data obtained from a model of renal ischaemia-reperfusion [9] suggested that Clusters 1 and 6 expressed resident signatures, while Cluster 5 expressed an infiltrative profile ([Supplementary data](#), Fig. S5).

MPC clusters spontaneously rearranged according to Control or RM-AKI condition (Fig. 1F). Trajectory analysis of MPC clusters allowed to identify two major trajectories composed of Clusters 1, 6, 7 and 5 and Clusters 2, 8, 0 and 3 in the transition from the Control to the RM-AKI condition (Fig. 1G).

Cluster 8 is a transition node between MHCII^{high} and MHCII^{low} MPC clusters

We next focused on macrophage Cluster 8 positioned at the junction between Control Cluster 2 and the most abundant RM-AKI associated clusters (0 and 3). Differential expression genes analysis of Cluster 8 between Control and RM-AKI revealed that the top downregulated genes were those of the major histocompatibility complex (MHC) class II (MHCII) genes family (*H2Eb1*, *H2-Ab1* and *CD74*) (Fig. 2A). We therefore compared the MHCII genes (*H2Eb1*, *H2-Ab1*, *H2A-a* and *CD74*) between all MPC clusters and we observed that their expression was higher in Control clusters compared with RM-AKI clusters (Fig. 2B and C). Control and RM-AKI clusters could therefore be classified as MHCII^{high} and MHCII^{low}, respectively. MHCII genes co-expressed with *Adgre1* in Control clusters, whereas RM-AKI clusters which did not express MHCII genes expressed *Itgam* ([Supplementary data](#), Fig. S4B). These observations showed that the previously identified R2 to R1–R0 switch in RM-AKI was accompanied by a downregulation of MHCII transcripts.

Interestingly, Cluster 8 expressed a bi-modal expression of the MHCII transcripts (Fig. 2B). Pseudotime analysis revealed Cluster 8 as a node between Cluster 2 (MHCII^{high} MPC) and Clusters 0 and 3 (MHCII^{low} MPC) (Fig. 2D). The down regulation of MHCII transcripts along the trajectory appeared to be a continuous process (Fig. 2E). We hypothesized that Cluster 8 could act as a transition cluster encompassing MHCII^{high} cells in homeostatic conditions that reprogram into MHCII^{low} cells under injury. Of note, Cluster 2 and Clusters 0 and 3 differed in term of gene expression for no less than 239 genes ([Supplementary data](#), Tables S1 and S2). Cluster 2 was enriched in antigen processing and presentation pathways, whereas Clusters 0 and 3 were e.g. enriched in oxidative phosphorylation, cytokine and cytokine receptor pathways ([Supplementary data](#), Fig. S6A and B).

Cluster 8 is enriched in both cell-cycle and cellular senescence genes

KEGG analysis revealed that Cluster 8 was enriched in 'Cell cycle' and 'Cellular senescence' genes when compared with the other MPC clusters (Fig. 3A). Similarly, exploration of simultaneous

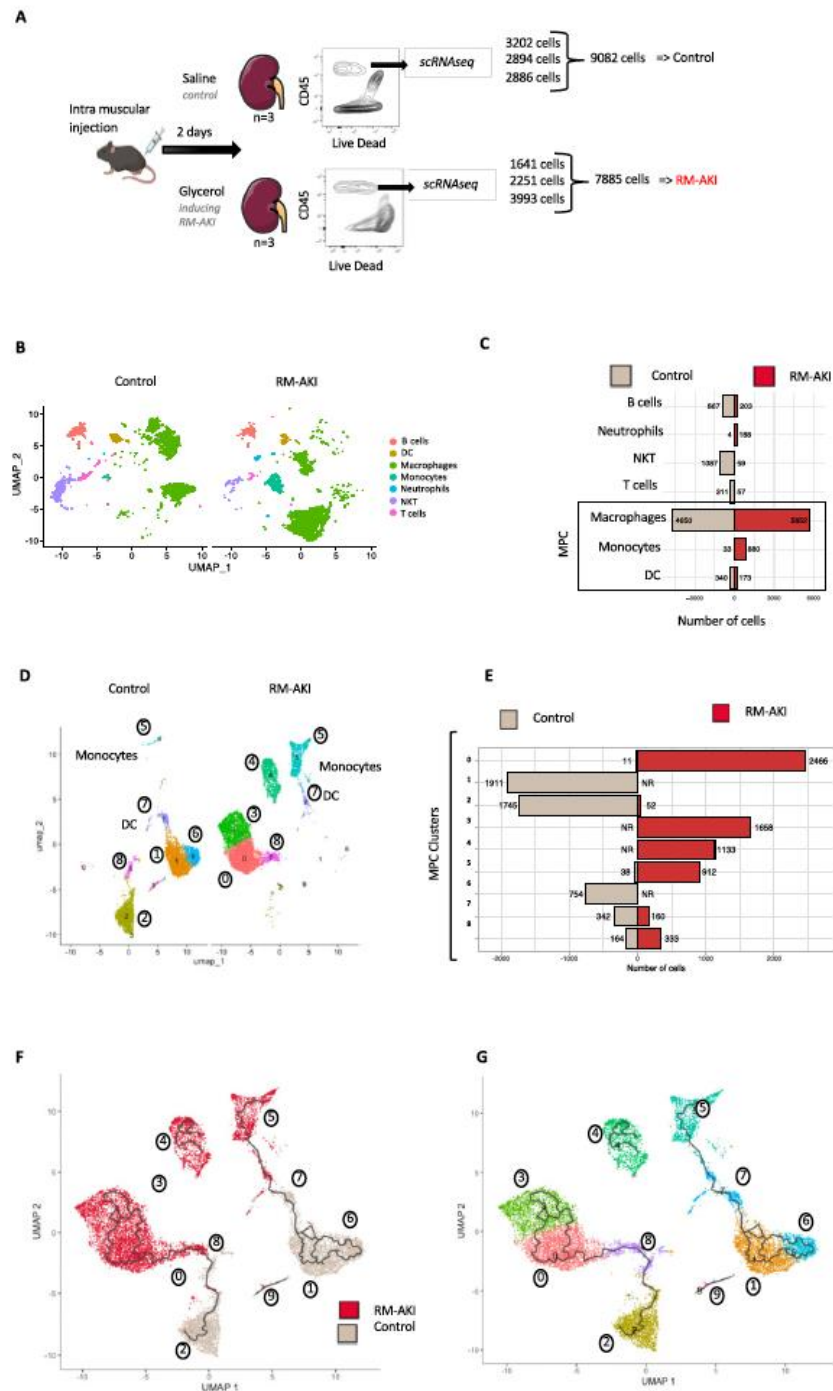


Figure 1: Immune cell diversity identified by scRNASeq analysis. (A) Schematic diagram representing experimental procedure where three mice were injected with saline as control (Control) and three mice were injected with 50% glycerol for induction of rhabdomyolysis (RM-AKI). (B) UMAP plot of scRNASeq data revealing distinct clusters in Control and RM-AKI conditions. Cells were identified as macrophages, monocytes, T cells, dendritic cells (DC), B cells, neutrophils and NKT using ImmGen datasets. (C) Bar graph indicating cell number of annotated immune cell populations in the Control and RM-AKI condition. (D) UMAP plot from scRNASeq data revealing subclustering of MPCs containing macrophages, monocytes and DC. Cells were annotated into nine clusters (0–8), where Cluster 5 was specific for monocytes and Cluster 7 for DC, while the rest of the clusters were for macrophages. Cluster 9 had a very low number of cells and was not considered further for analyses. (E) Bar graph indicating the cell number of the respective annotated clusters in Control or RM-AKI condition in MPC clusters. (F) UMAP plot of the MPC clusters showcasing the segregation of clusters according to Control or RM-AKI condition. (G) Trajectory plot indicating the spatial rearrangement of MPC clusters.

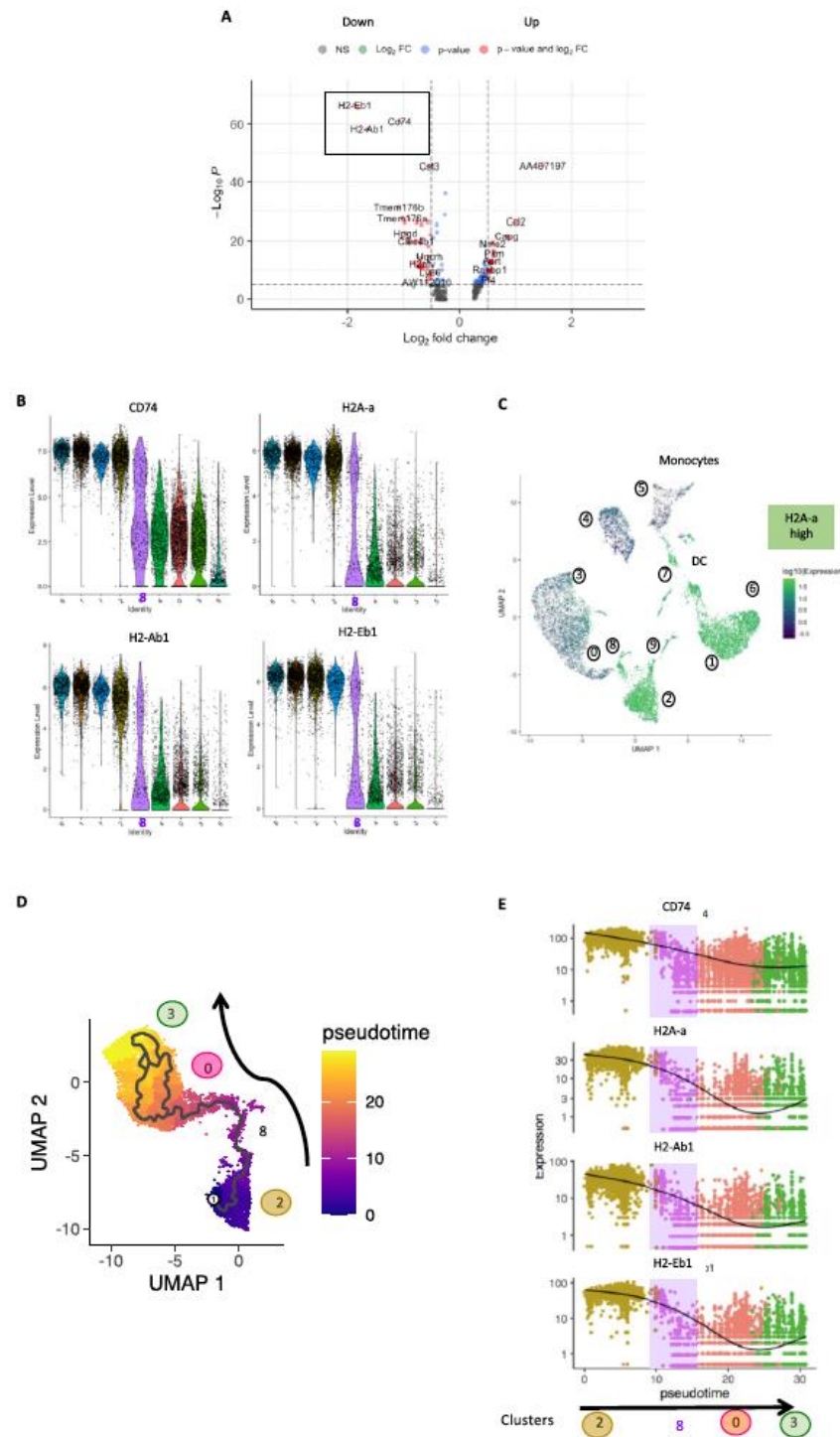


Figure 2: Cluster 8 as a transition node between MHCII^{high} and MHCII^{low} MPC clusters. (A) Volcano plot indicating differentially expressed genes in Cluster 8 after RM-AKI: down-regulated genes on the left and up-regulated genes on the right. In the black rectangle, top genes significantly down-regulated were MHCII transcripts. (B) Violin plot illustrating SC expression of MHCII markers (H2Eb1, H2-Ab1, H2A-a and CD74) in MPC clusters. Cluster 8 is the only cluster with a bimodal expression of MHCII markers. (C) An example of a MHCII gene expression (H2A-a) in each cell in the UMAP representation of MPC clusters. (D) Pseudotime plot that was generated by fixing startpoint on the trajectory in Cluster 2. (E) Normalized expression of MHCII markers (H2Eb1, H2-Ab1, H2A-a and CD74) throughout the pseudotime trajectory on cluster path 2, 8, 0 and 3. Cells are colored by clusters.

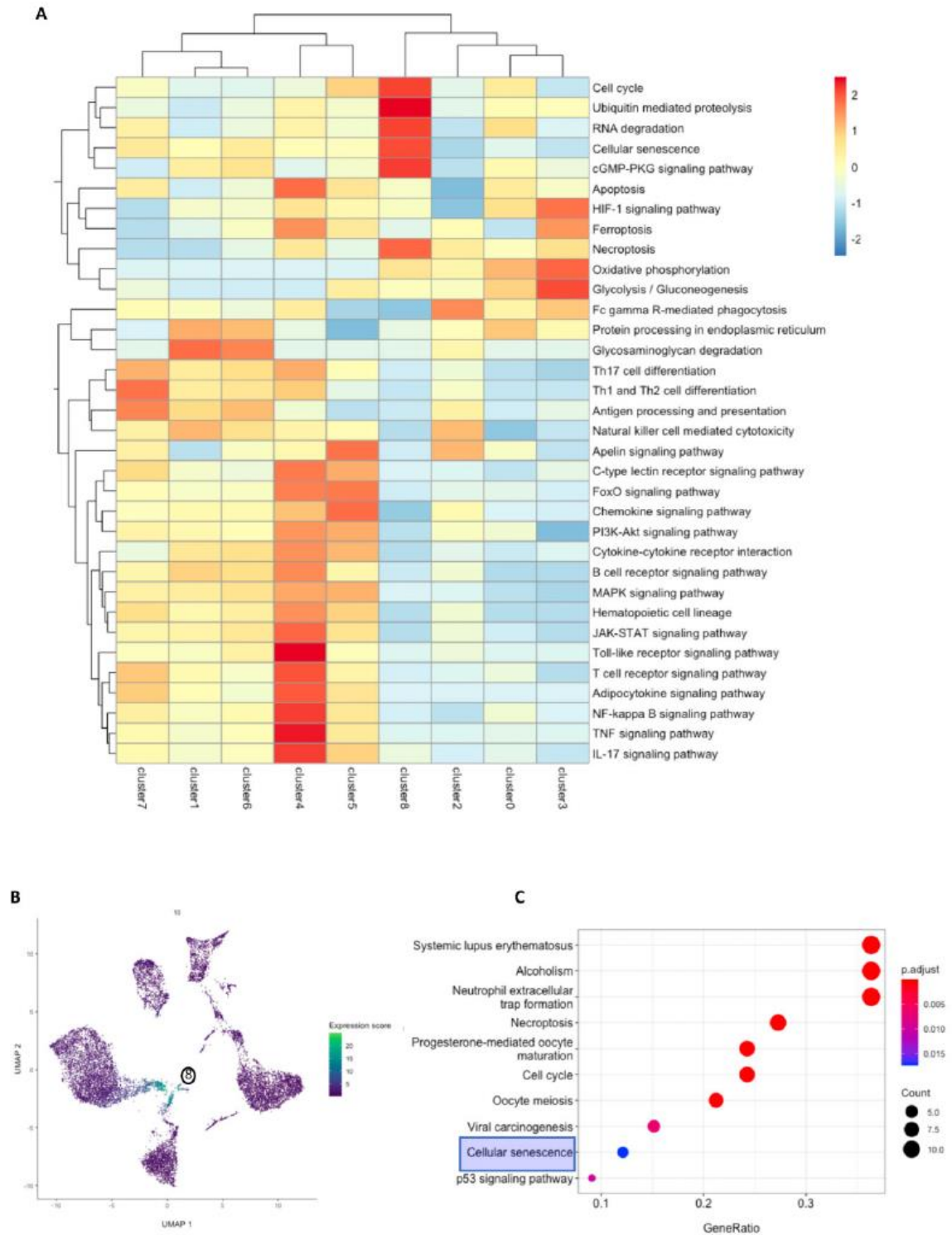


Figure 3: Cluster 8 is enriched cell cycle and cellular senescence genes. (A) Heatmap representing 34 enriched KEGG pathways across the UMAP MPC clusters, scaled by row. The EnrichKEGG function was run for every cluster on its gene markers and with all genes expressed in all clusters as background. Non-significant enrichments (Padjusted $\geq .05$) were set to 0. (B) UMAP plot of the mean expression of a module of genes in each cell. This module is specifically expressed in Cluster 8. (C) Dot plot showcasing the top 10 enriched KEGG pathways of the genes in the module defined in (B).

genes expression along the trajectories in a non-supervised manner revealed two modules including 'Cell cycle' and 'Cellular senescence' specifically enriched in the Cluster 8 region (Fig. 3B and C). Finally, the Reactome enrichment analysis of Cluster 8 also revealed that 'Cell cycle' and 'Cellular senescence' pathways were predominant (Supplementary data, Table S3). These observations were consistent with the fact that the top up-regulated genes specific of Cluster 8 (Table 1) were: (i) *Stathmin 1*, which is associated with microtubule filament system; (ii) *Ube2c* in cell cycle; (iii) *Hist1h1b* in condensation of nucleosomes; (iv) *Birc5* in cell proliferation and preventing apoptosis; (v) *Hist1h2ae* core component of nucleosome; and (vi) *Pclaf* coding for a regulator of DNA repair and centrosome number. Cluster 8 had the highest expression of: *Tubb5* and *Tuba1b* coding for proliferation-related proteins, *H2afz* coding for histone protein, and *Hmgb2* involved in DNA bending and circling. Taken together, these data indicate that Cluster 8 could play a role in MPC renewal during injury. Of note, the cell proportion in Cluster 8 doubled between Control and RM-AKI condition (Fig. 1E), suggesting a possible proliferation of this cluster during RM-AKI according to 'Cell cycle enrichment'.

TECs also express cellular senescence genes during AKI

Because injured tubular epithelial cells (TECs) are known to interact closely with infiltrating immune cells, we wished to investigate whether cellular senescence was also occurring in TECs compartment, and whether the TECs-senescence was identical to the MPCs-senescence signature. KEGG analysis for 'Cellular senescence mmu04218' was performed on public single-cell or single-nuclei datasets obtained from murine models of AKI [6–8] (Fig. 4A, details in Supplementary data, Table S4). Cellular senescence signature was statistically enriched in TECs, especially proximal tubule S3 and S1. Principal cells, loop-of-Henlé, distal convoluted tubule and intercalary cells also exhibited senescence pattern (Fig. 4B). A total of 75 genes were found to be enriched in TECs (Fig. 4C). Except for *Rbbp4* (RB Binding Protein 4, Chromatin Remodeling Factor), none of the TECs enriched genes was common with MPC Cluster 8, implying that cellular senescence takes a different form depending on which renal compartment is studied. Because senescence had never been studied in RM-AKI samples, and because *Cdkn1a* (corresponding to p21) was found to be enriched in TECs signature, we looked at its expression in bulk kidney tissues (Fig. 4D and E). Both p21 and *Fgf2* Fibroblast Growth Factor (causing a failure of cell cycle progression) were upregulated 2 days after RM-AKI, suggesting that senolytics could be efficient in preventing RM-AKI lesions.

Senolytics DQ are nephroprotective in RM-AKI

The expression of a gene network related to cellular senescence raised the hypothesis that senolytics could be a pharmacological tool to target both TECs early senescence and blocking the MPC eflux, thereby reducing RM-AKI related damages. Combined dasatinib (D, a tyrosine kinase inhibitor) and quercetin (Q, a flavonol that inhibits PI3K) treatment had been reported to reduce the senescent cell burden and renal fibrosis in both unilateral renal ischemia reperfusion and cisplatin-induced murine AKI [10]. Consequently, we assessed whether a combined treatment with DQ could counteract RM-AKI. DQ or its vehicle was administered daily, 3 days before and 2 days after glycerol injection to induce RM-AKI in mice (Fig. 5A). DQ did not modify plasma creatinine phosphokinase (CK) at 6 h compared with vehicle, indicating

no influence of DQ on RM. In contrast, DQ significantly reduced blood urea nitrogen (BUN) at 48 h compared with vehicle, indicating a prevention of RM-AKI (Fig. 5B). DQ treatment attenuated the RM-AKI-induced injury associated genes expression pattern [*KIM-1*, *TNF- α* , *TGF- β* , *HO-1* and *p21* overexpression, and Transmembrane protein 27 (*TMEM-27*) corresponding to collectrin expected downregulation] [11] (Fig. 5C). Interestingly, DQ treatment reverted the phenotypic switch in MPC fractions analysed through conventional cytometry after RM-AKI. The R2/R1 ratio, drastically decreased in the RM-AKI condition, was significantly ($P < .01$) reversed by DQ treatment (Fig. 5D and E). DQ treatment did not affect other explored immune cells (B cells, neutrophils; except CD4/CD8 T cells ratio, which was increased by DQ) (Supplementary data, Fig. S7A). DQ treatment in control mice had no effect on CPK, BUN or on immune cells (Supplementary data, Fig. S7B and C). Analyses of kidney structure revealed a trend in attenuated lesions (measured by tubular injury score) and apoptosis detection (Supplementary data, Fig. S7D). Because senolytics could have only delayed AKI occurrence, we also studied a later timepoint, Day 7, after RM induction (Fig. 6A). DQ effect was confirmed over time, with a significant improvement of kidney function (Fig. 6B). R2/R1 reversal was sustained at Day 7 as well (Fig. 6C and examples shown in Supplementary data, Fig. S8A). Survival was between 80% and 100% (Supplementary data, Fig. S8B).

Taken together, these data suggest that a senolytics combination preserves kidney function when used at an early stage of RM-AKI, with a sustained effect.

DISCUSSION

In summary, our study presents the first analysis of the single-cell transcriptional signature of kidney immune cells in RM-AKI in mice and provides an *in vivo* proof-of-concept that senolytics can be protective at the early phase of RM-AKI.

Genetic fate-mapping combined with parabiosis and adoptive transfer strategies revealed that macrophage dynamics may originate from (i) embryonic precursors that differentiate into self-renewing tissue-resident macrophages and (ii) bone marrow-derived haematopoietic stem cells that differentiate into blood monocytes [12, 13]. Gene expression between $MHCII^{high}$ and $MHCII^{low}$ cells helped to identify additional MPC dynamics. We observed divergent transcriptional programming of MPC subpopulations after injury. We confirmed the data obtained after renal ischaemia-reperfusion that favour a transcriptional reprogramming of resident MPC toward a developmental state characterized by a downregulation of $MHCII$ [14].

Importantly, we identified for the first time a cluster, Cluster 8, encompassing both $MHCII^{high}$ cells in the Control condition and $MHCII^{low}$ cells in the RM-AKI condition. We propose that Cluster 8 behaves as a reprogramming node between Cluster 2 and Clusters 0 and 3. While quite small, this cluster doubled in RM-AKI conditions, and appeared as the potential source of intrarenal MPC Clusters 0 and 3. Genes in Clusters 0 and 3 are involved in processes such as ferroptosis and necroptosis, two inflammatory events known to be involved in RM-AKI [15]. We hypothesized that inhibiting Cluster 8 could impact on Clusters 0 and 3, therefore decreasing the inflammatory burst that could lead to kidney injury during RM. Surprisingly, cells from Cluster 8 expressed a dense network of 'Cell cycle' and 'Cellular senescence' genes (Fig. 7). Cellular senescence is a long-lasting cell cycle arrest that helps cells to avoid uncontrolled mitosis providing more time for DNA repair. We believe that Cluster 8 represents 'premature senescence' that

Table 1: Characteristics of MPC cluster.

MPC cluster	Cell type	% of MPC in control	% of MPC in RM-AKI	Adgre 1	Itgam	F4/80 CITE Seq	MHCII	Literature-based signature	M1 markers	M2 markers	Enriched genes in DEG analysis (one cluster compared with all the others)
0	Macrophages	0.2	36.7	x	x	Int			Ccl2		Eif5a, Gapdh, Nme1, Cotl1, Ccl12, Bcl2a1d, Bcl2a1a
1	Macrophages	38.5	0	High	x	High	High	Resident			Cx3σ1, Malat1, Fyb, Ptprc, Tgfbf1, Mbnl1, CD74
2	Macrophages	35.1	0.8	High	x	High	High		Irf5, Ccl2		Aif1, Ist1, Wfdc17, Phf11, Hpgd
3	Macrophages	0	24.7	x	x	Int					Mt1, Mt2, Fth1, Pf4, Lgals1, AA467197
4	Macrophages	0	16.9	x	High	High/int			Ccl2, Ccl3, Tlr2, Ilr4ra, Nfkbiz, Cybb		Cxcl2, Ccl3, Ccl4, Junb, RGS1, Dusp1, Socs3, Fos
5	Monocytes	0.8	13.6	x	High	Low		Infiltrating	Hif1a, Tlr2, Irf5, Clec4e, Ilr4ra, Nfkbiz, Ccl2, Cybb	Klf4, Hmox1, vcan, Thbs1, Fosl1, Plaur	Itgb2, Ccl9, Irf7, Ccr1, Fn1 a priori search R1/S100a8, Cebpβ, Vim, Ccl2
6	Macrophages	15.2	0	High	x	High	High	Resident			Cx3σ1, Klf2, Mafk, Jun, Jund, ier5
7	Dendritic cells	6.9	2.4	x	x	Low			Irf5, Ilr4ra, Nfkbiz, Ccl2, Cybb	Klf4	Cd209a, Tnfp3, Klrd1, Lsp1
8	Macrophages	3.3	5	x	x	Int	Bimodal		Ccl2, Ciita		Stathmin 1, Ube2c, Hist1h1b, Birc5, Hist1h2ae, Pclaf, Tubb5 and Tuba1b, H2afz, Hmgb2

DEG, differentially expressed gene; Int, intermediate; X, absence (not present).

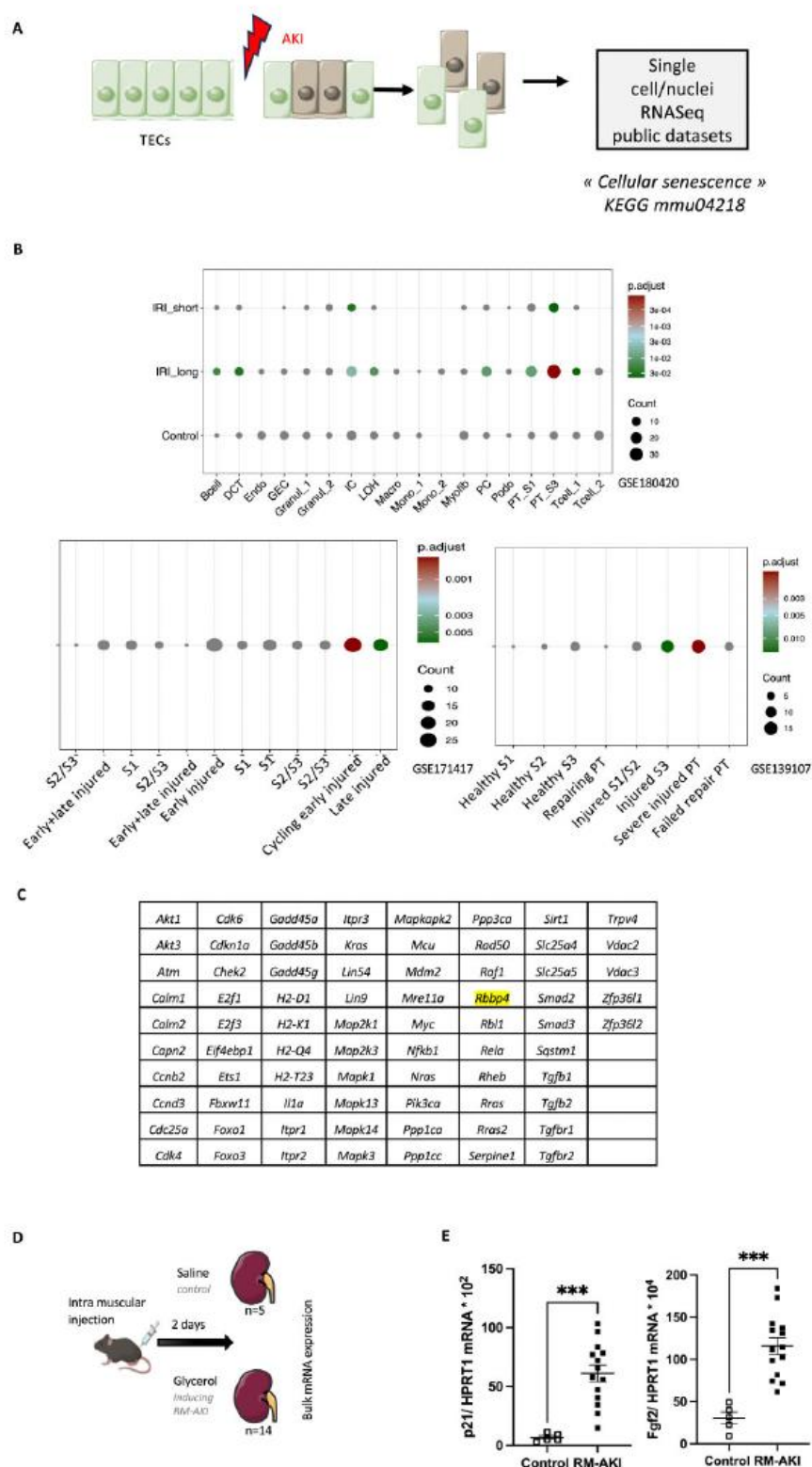


Figure 4: Cellular senescence analysis in TECs sorted from distinct AKI models. (A) KEGG mmu04218 cellular senescence signature was looked for in single cell/nuclei RNASeq datasets in TECs. (B) The enrichment of the signature is depicted in the AKI scRNASeq literature obtained clusters. (C) A total of 75 genes were found to be enriched in TECs. *Rbbp4* was the only common gene with Cluster 8 signature (highlighted). (D) RM-AKI was performed and bulk kidney tissue studied for mRNA expression. (E) *p21* and *Fgf2/HPRT1* mRNA are depicted in control mice and compared with RM-AKI mice at Day 2. *** $P < .001$.

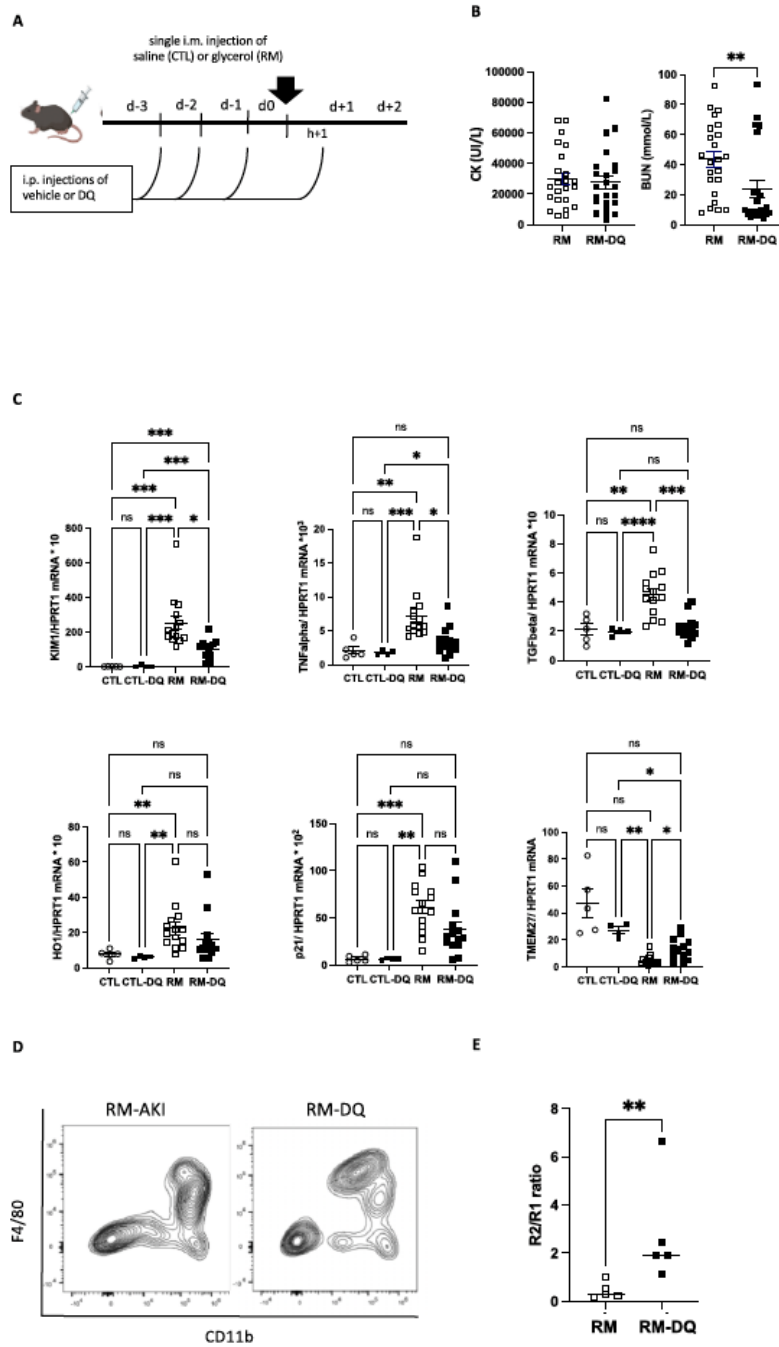


Figure 5: Senolytics effects on RM-AKI. (A) Schematic diagram representing the experimental procedure. (B) CK measurement in blood at 6 h after RM; $n = 23$ (RM-AKI) and $n = 23$ (RM-AKI with DQ treatment). BUN measurement in blood at 48 h after RM ($P < .05$); $n = 23$ (RM-AKI) and $n = 23$ (RM-AKI with DQ treatment). (C) mRNA expression/HPRT1 in bulk kidney tissue collected after 2 days in each group. KIM-1, kidney injury molecule 1; TNF- α , tumour necrosis factor-alpha; TGF- β , transforming growth factor beta; HO-1, heme oxygenase 1; p21, TMEM-27, corresponding to collectrin. * $P < .05$, ** $P < .01$, *** $P < .001$. (D) Illustrative example of MPC phenotypes obtained by flow cytometry. Dot plot of F4/80 vs CD11b gated on CD45 live cells obtained from digested kidneys. F4/80^{low}CD11b^{high} cells (R1) were dominant MPC in the RM-AKI condition. DQ treatment attenuated the proportion of R1 cells in favour of a higher proportion of F4/80^{high}CD11b^{low} cells (R2). (E) The R2/R1 ratio was obtained by calculating the ratio between F4/80^{high}CD11b^{low} cells (R2) divided by F4/80^{low}CD11b^{high} (R1) cells, gated on CD45 live cells. DQ significantly increased the R2/R1 ratio ($P < .05$); $n = 5$ (RM-AKI) and $n = 5$ (RM-AKI with DQ treatment).

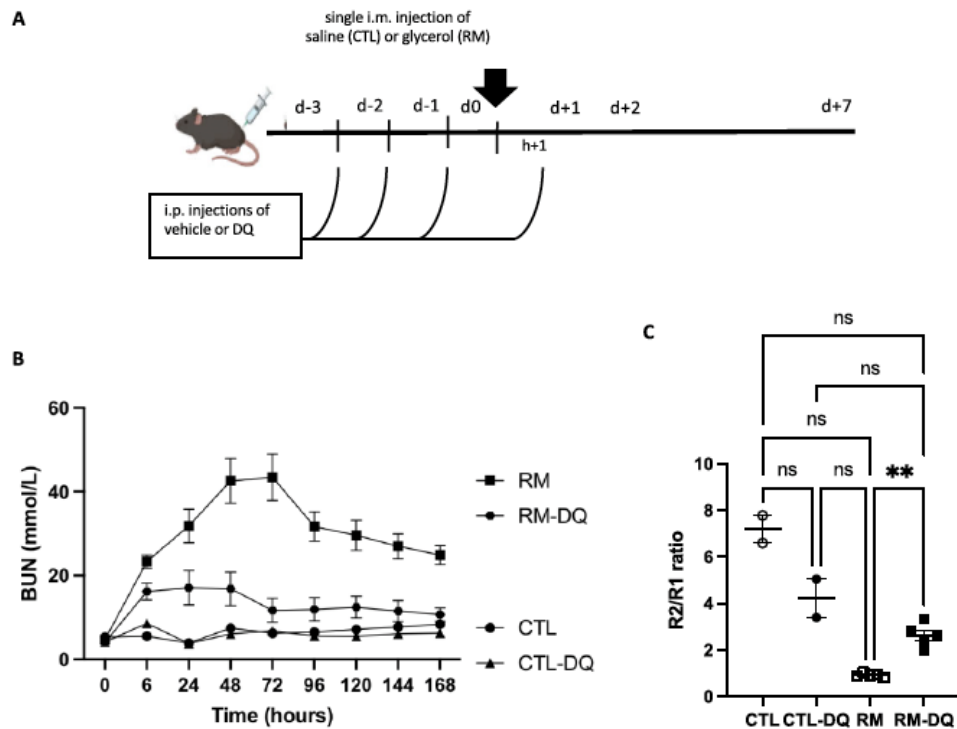


Figure 6: Senolytics effect is sustained after 7 days. (A) Schematic diagram representing the experimental procedure. (B) BUN measurement in blood expressed according to time in hours. Two-way ANOVA was significant ($P < .001$) for all comparisons except CTL vs CTL-DQ. CTL, control. (C) The R2/R1 ratio was obtained by calculating the ratio between F4/80high CD11b^{low} cells (R2) divided by F4/80^{low} CD11b^{high} (R1) cells, gated on CD45 live cells. DQ significantly increased the R2/R1 ratio ($p = 0.0070$), $n = 5$ (RM-AKI) and $n = 5$ (RM-AKI with DQ treatment) while no significant change was observed due to DQ treatment in control $n = 2$ (CTL) and $n = 2$ (CTL with DQ treatment).

occurs during acute organ injury rather than 'replicative senescence' due to chronological aging. For the first time we found such 'cellular senescence' genes in MPC populations in young mice and at an early phase of AKI, independently of ageing processes. Whether AKI leads to premature ageing of immune cells would deserve further studies. Furthermore, a spatial analysis of senescent Cluster 8 would have been of great added value to our study. However, Cluster 8 represents a small subpopulation of the MPCs (333 cells out of 6714, i.e. 4.9%). Therefore, Cluster 8 spatial localization would be very difficult to assess unless using a single-cell spatial transcriptomic approach.

Because it has been proposed recently that cellular senescence could be immunogenic [16], Cluster 8 senescent cells may represent an aberrant signal that activates the adaptive immune system, which in turn would amplify a harmful immune response. Blocking cellular senescence processes occurring in Cluster 8 could lead to an attenuation of the MPC afflux. Importantly, cellular senescence is not restricted to immune cells compartment, as assessed by the analyses of TECs scRNASeq and single-nuclei RNASeq in AKI contexts. Although the gene signature is not identical, cellular senescence processes are likely to occur at early phase of AKI in a ubiquitous fashion.

Senolytics are responsible for the elimination of senescent cells through targeting senescent cell antiapoptotic pathways [17]. The

first generation of senolytics included the DQ combination: dasatinib is a tyrosine kinase inhibitor acting through inhibition of Ephrin B-dependent suppression of apoptosis and is an already Food and Drug Administration-approved drug for treatment of chronic myeloid leukemia (NDA #021986 and 022072). Quercetin causes cell death through inhibition of phosphoinositide 3-kinase [17]. The senolytic effects of DQ have been reported in cell lines as well as in murine models of variety of diseases including Alzheimer's, fibrotic pulmonary disease and age-dependent hepatic steatosis [18–20]. DQ treatment has been reported to reduce senescent cell burden in renal unilateral ischaemia-reperfusion injury and multiple-cisplatin-treatment murine models [10]. Similarly, a reduction in kidney fibrosis post-ischaemic AKI was observed after DQ [10]. However, the effects of DQ treatment on RM-AKI have never been addressed and senescence of immune cells at the early phase of the injury has not yet been studied.

In conclusion, our study demonstrates the heterogeneity of immune cells and MPC induced after RM-AKI and that immune cells express a cellular senescence signature which is distinct from *in silico*-obtained TECs' signatures. Further studies are needed to demonstrate the distinct role of each compartment (immune vs TECs) in senolytics nephroprotective effects.

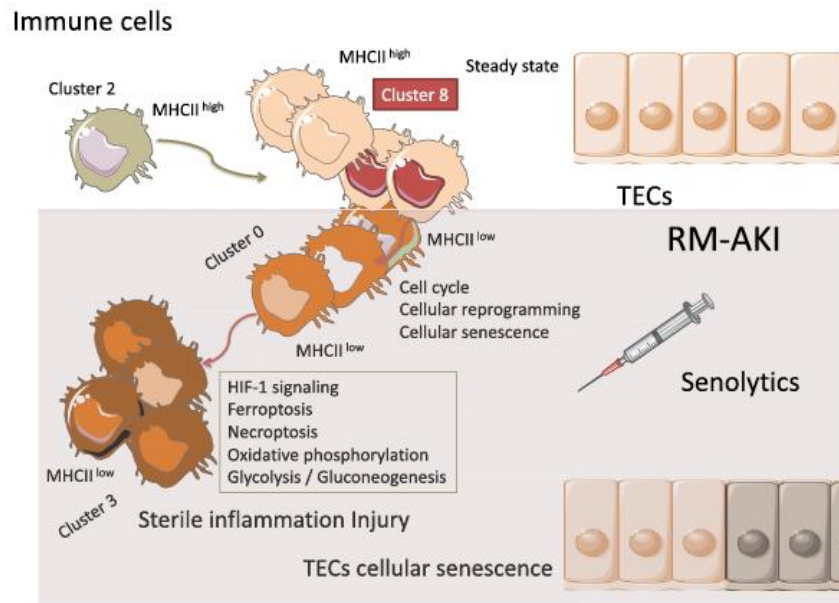


Figure 7: Theoretical mode of action of senolytics. Senolytics could act both on TECs and MPC reprogramming during AKI.

SUPPLEMENTARY DATA

Supplementary data are available at [ndt](https://ndt.oxfordjournals.org/) online.

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AUTHORS' CONTRIBUTIONS

S.N.R. performed RM-AKI experiments and DQ treatments, performed the data analysis and wrote the initial version of the manuscript. M.Z. performed single-cell data analyses. M.B. and A.C. performed RM-AKI experiments. S.F. revised the manuscript. G.F. performed RM-AKI experiments. J.S.I. supervised preliminary single-cell analyses. O.P.J. contributed to the interpretation of the results and provided critical feedback and helped shape the analysis and manuscript. I.G.-F. performed initial single-cell data analyses. E.L. and F.M. were involved in the 10X experiments. E.R. and A.Z.-G. performed cell sorting. J.B., J.P.S. and J.S.S.B. designed the study and were in charge of overall direction, planning and manuscript writing.

DATA AVAILABILITY STATEMENT

Sequencing data has been deposited on the Gene Expression Omnibus repository (GSE227123). To review the sequencing data go to <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE227123> and enter token alwdqqouburpen into the box.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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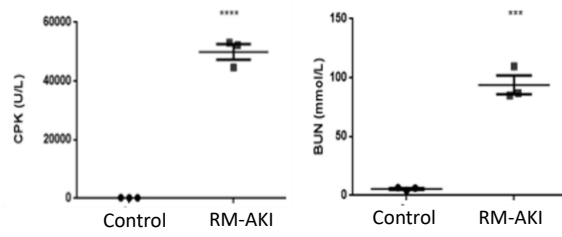
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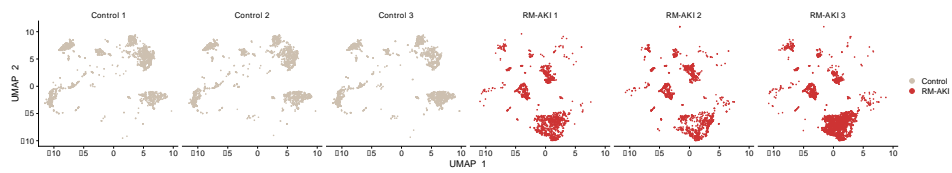
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Supplementary Figure

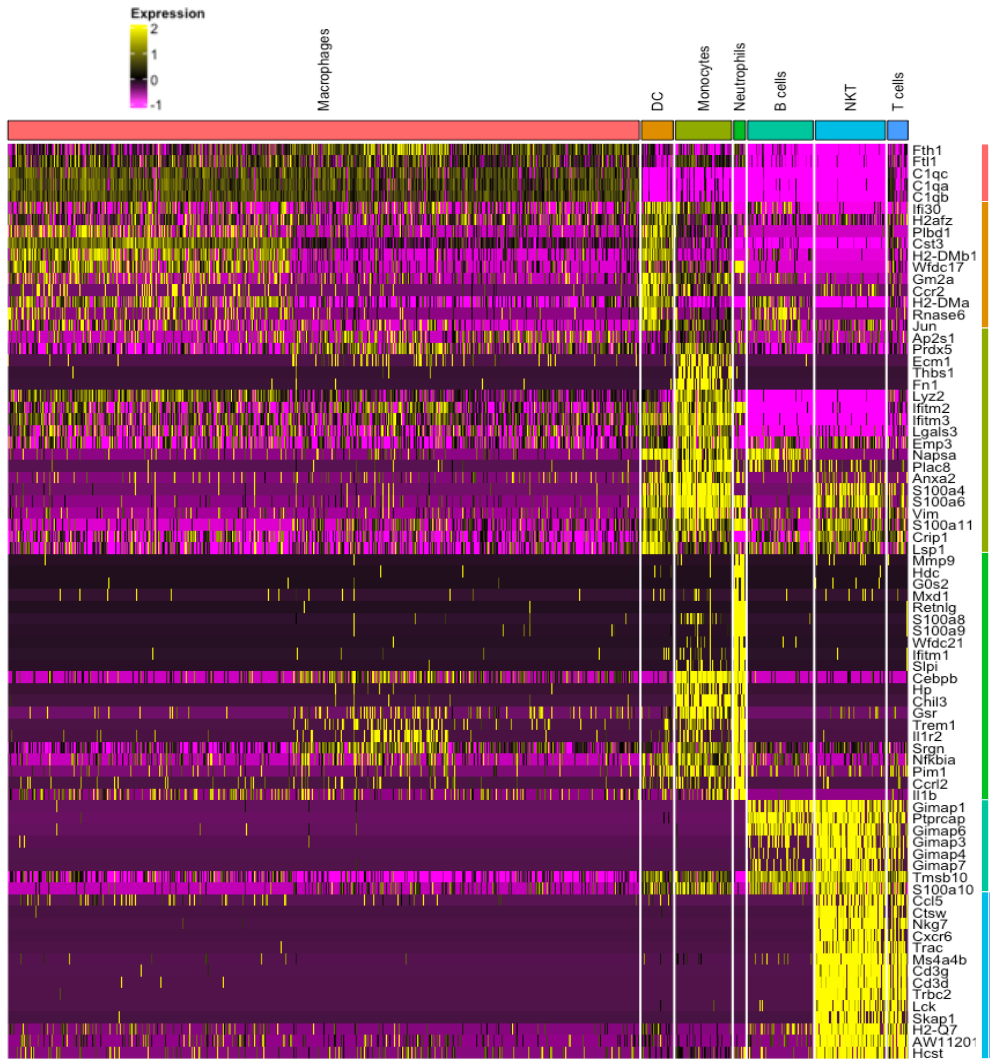
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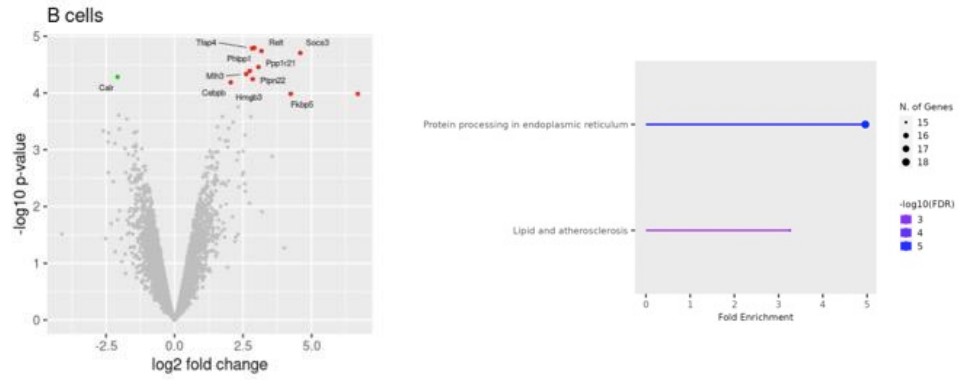


Suppl Figure 1

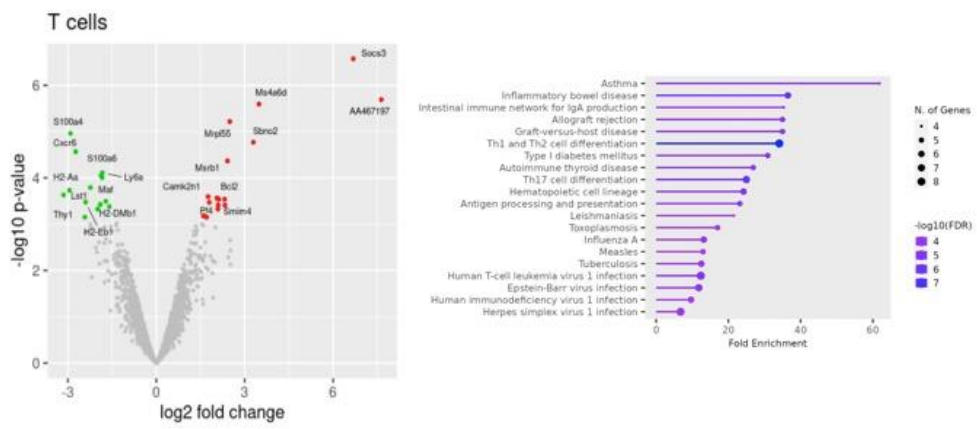


Suppl Figure 2

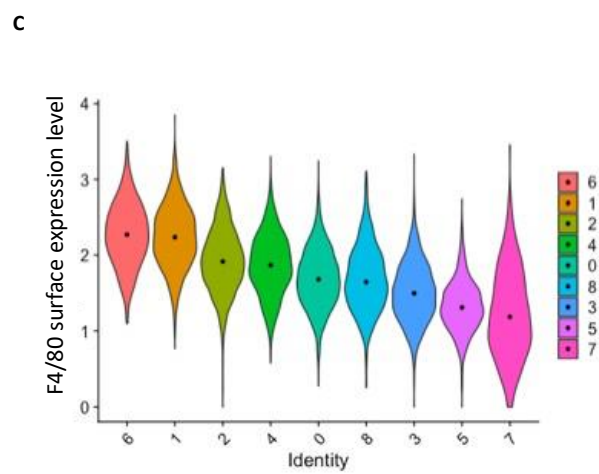
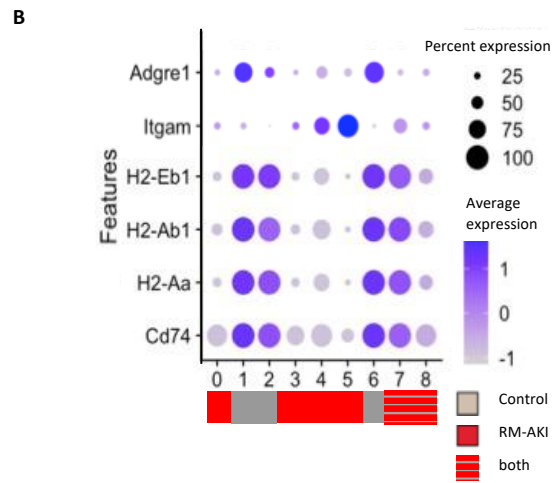
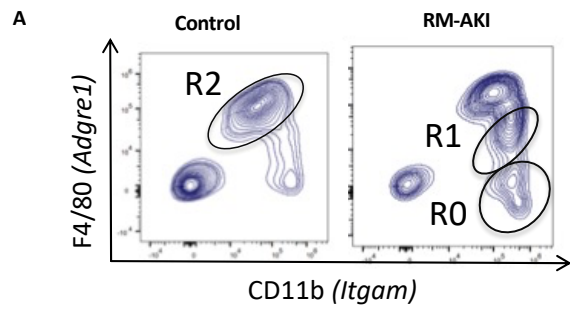
A

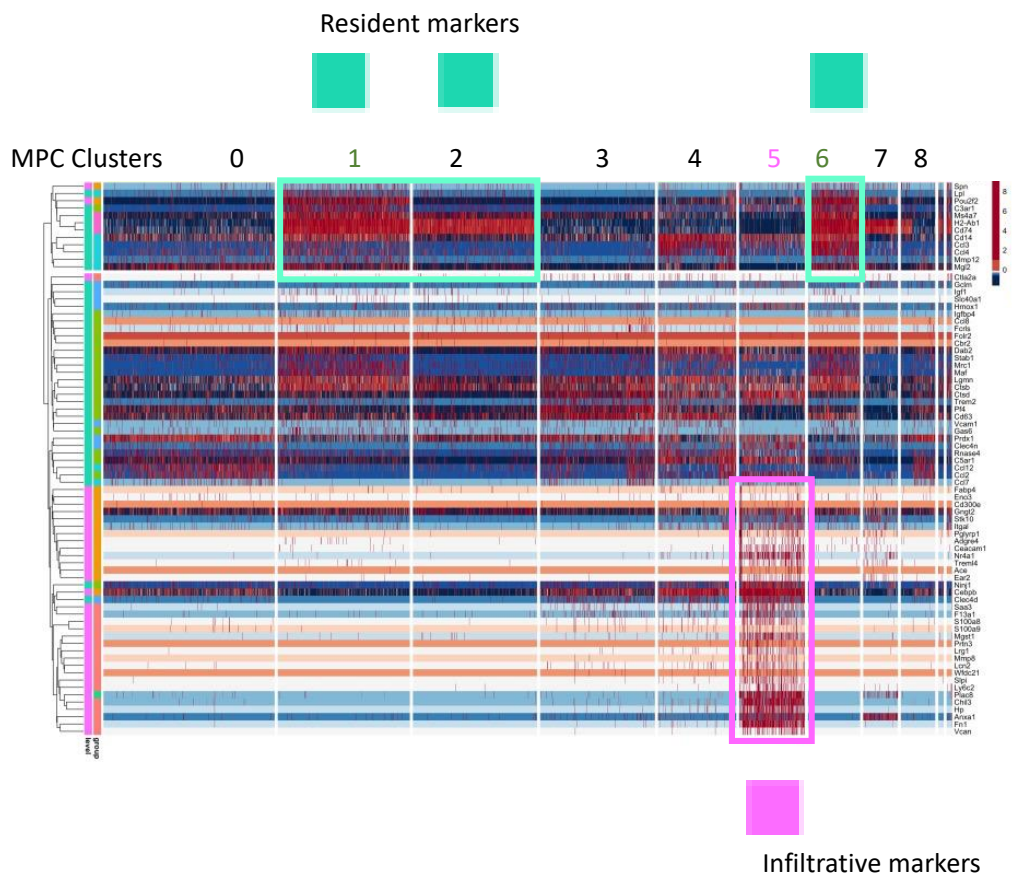


B



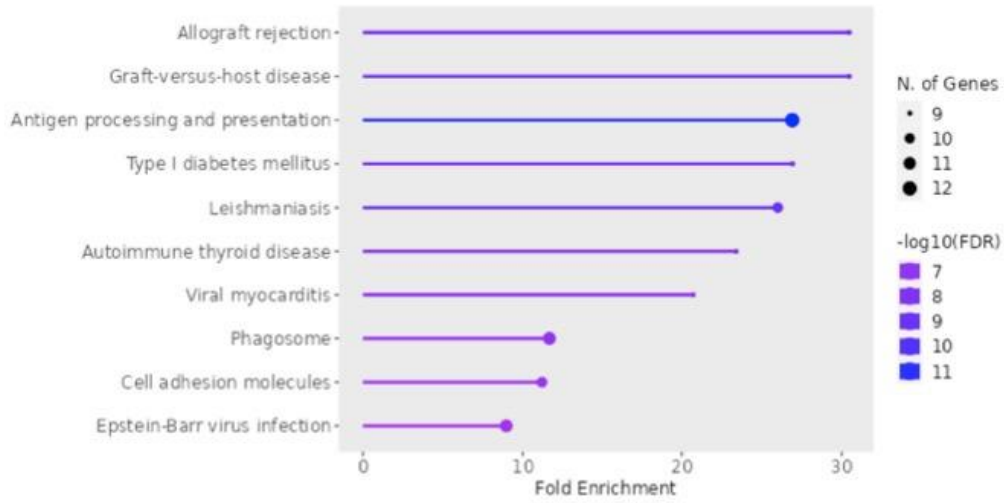
Suppl Figure 3





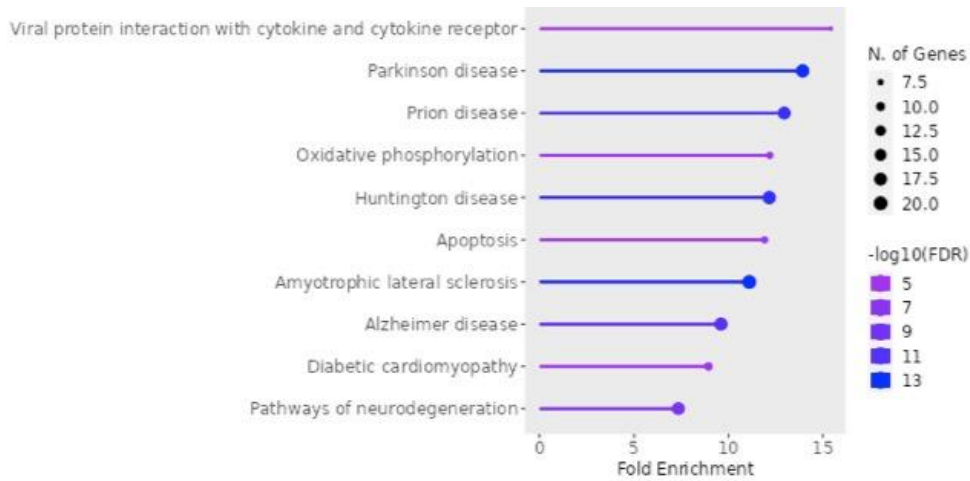
A

Upregulated genes in MPC cluster 2 compared to MPC clusters 0 and 3



B

Downregulated genes in MPC cluster 2 compared to MPC clusters 0 and 3

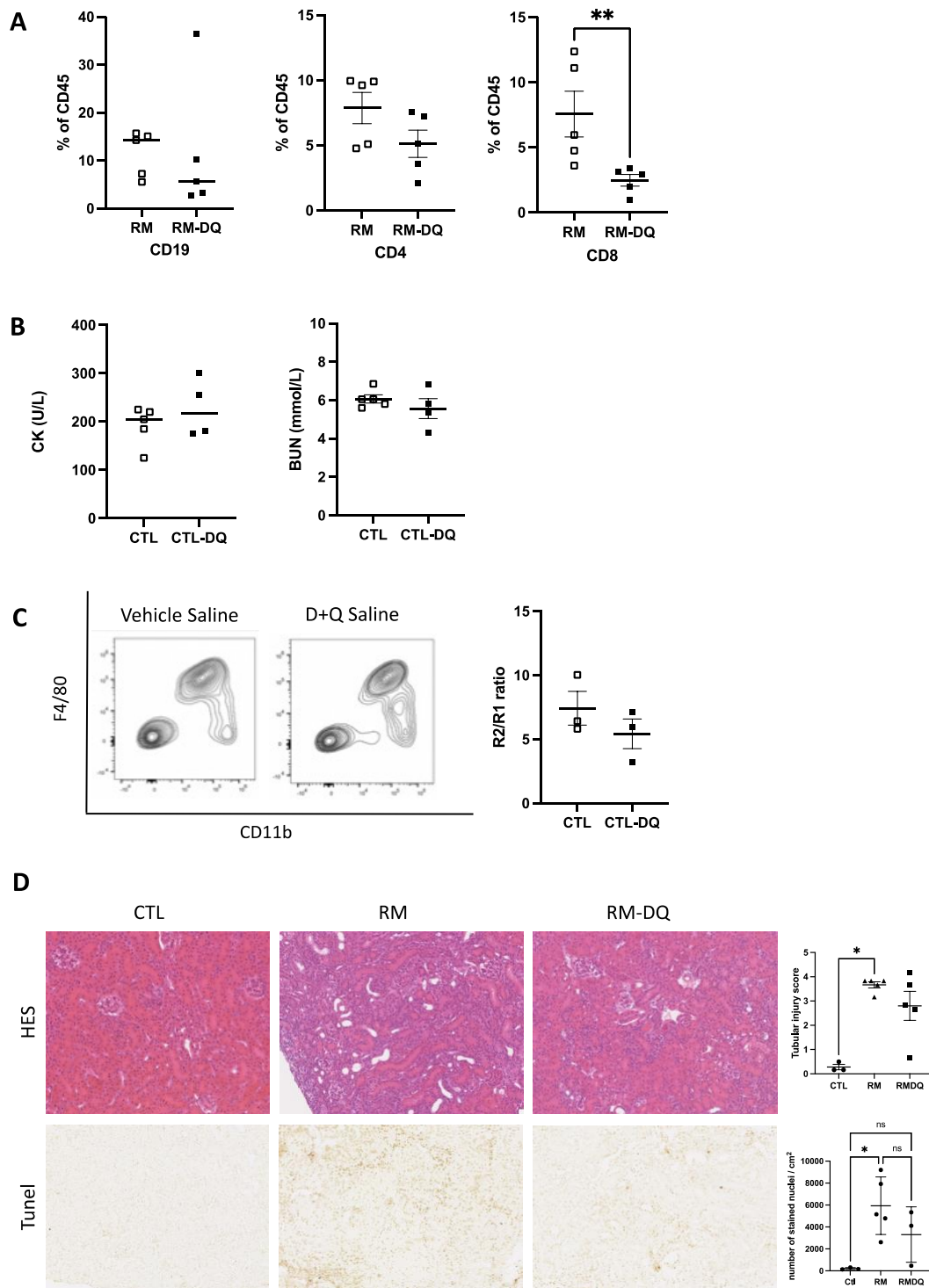


A

GSE number	Type of data	AKI model	Time points	Cluster legend
GSE139107	Single nuclei	UUO	Control 4 hours 12 hours 2 days 14 days 6 weeks	1. Healthy S1 2. Healthy S2 3. Healthy S3 4. Repairing PT 5. Injured S1/2 6. Injured S3 7. Severe injured PT 8. Failed repair PT
GSE171417	Single nuclei	IRI	7 days 28 days	0. S2/S3 1. Early+late injured 2. S1 3. S2/S3 4. Early+late injured 5. Early injured 6. S1 7. S1 8. S2/S3 9. S2/S3 10. Cycling early injured 11. Late injured
GSE180420	Single cell	IRI-short 23 minutes IIRI-long 30 minutes	1 day 3 days 14 days	

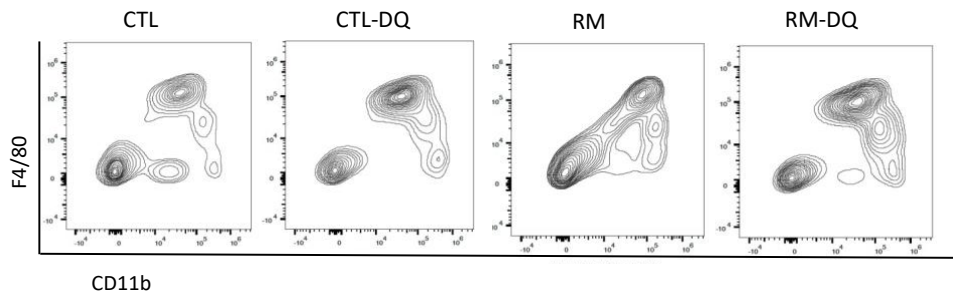
B

data set	UUO - GSE139107		IRI - GSE171417				IRI - GSE180420									
	nb of genes	13	7	13	26	19	31	16	15	19	10	8	10	13		
cell types enriched	Injured S1	Severe injured PT	S2/S3	S1	Cycling early injured	Late injured	PT_S3_IRI long	IC_IRI long	PC_IRI long	PTS1_IRI long	LOH_IRI long	IC_IRI short	DCT_IR long	PT_S3_IRI short		
genes	Cdkn1a Cdk6 Myc Serpin1 Mdm2 Smad3 Nras E2f3 Map2k3 Mapkapk2 Mapk14 Fbxw11	Gadd45g Gadd45a Sqstm1 Mdm2 Cdkn1a Ppp3ca Raf1 Map2k3 Mapk14 Sir1 Rbbp4 Mapkapk2 Pik3ca Myc Fbxw11	Itpr1 Foxo1 Rras2 Zfp36l1 Cdk6 Nfkb1 Itpr2	Itpr2 Gadd45g Foxo3 Fovw3 Capn2 Smad2 Smad3 Akt3 Zfp36l1 Rras2 Cdk6 Nfkb1	Rbl1 E2f3 Lin54 Akt3 Rbbp4 Rela Tgfb2 Ras Chek2 Calm2 Mapkapk2 Mre11a Capn2 Mcu Mapk1 Cdk25a Smad2 Nfkb1 H2-Q4 Trpv4 Calm2 Kras Mapk14 Itpr1	Nfkb1 Zfp36l1 Ets1 Smad3 Ets1 Kras Mapk14 Itpr1	Cdkn1a Gadd45b H2-D1 Calm1 Zfp36l2 Calm2 Tgfb1 H2-K1 Ila Eif4ebp1 Mapkapk2 Mdm2 Rheb Ppp1cc Rras Akt1 Tgfb1 Nfkb1 Zfp36l1	Cdkn1a Gadd45b Rras Eif4ebp1 Mapk13 Rras H2-D1 H2-D1 Zfp36l2 Itrp3 Rheb Calm1 Ppp1cc Gadd45b Ppp1ca Tgfb1 Calm1 Vdac3 Nras H2-K1 Smad3	Cdkn1a Rras Mapk13 Cdkn1a H2-D1 Gadd45a Rras H2-K1 Zfp36l1 Ppp1cc Gadd45b Ppp1ca Vdac3 Vdac2 Vdac2 Ppp1ca Mapk3 Kras H2-T23 Gadd45a Nras Rela Itrp2 Cnd3 Sqstm1 Vdac3	H2-D1 H2-K1 Cdkn1a Rras Cdkn1a Gadd45a H2-D1 H2-K1 H2-T23 Zfp36l1 Gadd45b H2-K1 Eif4ebp1 Rheb Ppp1ca Mapkapk2 Sqstm1 Sic25a5	Tgfb1 Zfp36l1 Ppp1cc Cdkn1a Rras Gadd45a Eif4ebp1 Zfp36l2 Calm1 H2-D1 Gadd45b Rras Zfp36l1 Tgfb1 Calm2 H2-K1 Eif4ebp1 Rheb Ppp1ca Mapkapk2 Sqstm1 Sic25a5	H2-K1 H2-D1 Cdkn1a Rras Gadd45a H2-D1 Gadd45b Rras Zfp36l1 Tgfb1 Calm2 H2-K1 Eif4ebp1 Rheb Ppp1ca Mapkapk2 Sqstm1 Sic25a5	Zfp36l1 H2-D1 Cdkn1a Vdac3 Eif4ebp1 Vdac2 H2-K1 Rras Gadd45a Calm1 Tgfb1 Calm2 Eif4ebp1 Rheb Ppp1ca Mapkapk2 Sqstm1 Sic25a5	Sic25a5 Sic25a4 Vdac3 Vdac2 H2-K1 Rras Calm1 Calm2 Ppp1ca Eif4ebp1 Rheb Gadd45a Zfp36l1		

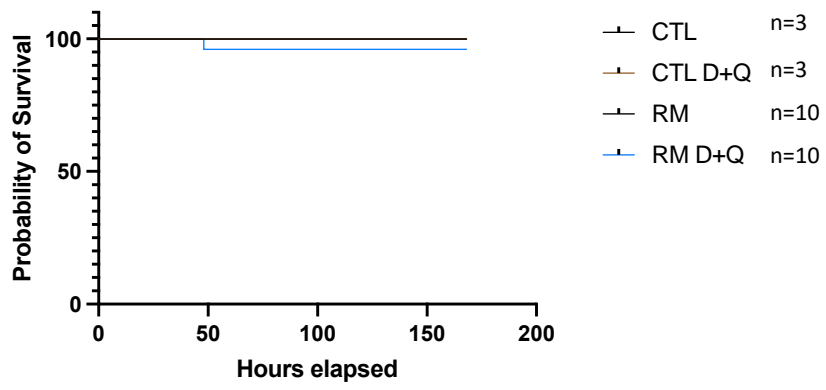


Suppl Figure 8

A



B



Manuscript 2 - Introduction

It is of increasing importance to analyse the effect of AKI in the elderly due to the increase in this population as well as the observed high incidence of worsened outcomes as well as increased mortality. Falls are responsible for 56.9% of RM cases and with at least 34% of the elderly experiencing a fall per year [39], fall risk is an important factor for RM-AKI in the elderly. In the following study, we wished to compare the effect of sex and age on the disease using an RM-AKI model system for fall risk.

RM was induced into two groups, young (2 months old) and old (26 months old) in male and female C57BL/6J mice and the effect was studied up to 37 days after RM induction. Through measurements of CK to assess the muscle damage and BUN to assess the renal function, sexual dimorphism was observed at a young age especially in males while females were observed to be more sensitive to RM-AKI with increasing age. qPCR and fibrosis markers were assessed highlighting the increased susceptibility of females to RM-AKI with age, leading to the hypothesis of females being a better model system to study the effect of RM-AKI with age. Variations in immune cell populations were observed as a trend both 2 and 37 days after RM induction.

Overall, this study brought to light the necessity of comparing the effect of aging and sex in association with RM-AKI.

MANUSCRIPT FOR SECOND PAPER

INTRODUCTION

Acute Kidney Injury (AKI) is becoming increasingly predominant reaching an incidence of 13.3 million cases per year and accounting for 1.7 million deaths per year. AKI is associated with an increase in long-term mortality [1, 2]. Multiple reports have showcased the higher incidence of AKI in the elderly (above 65 years of age) and illustrated an association between age and AKI [3]. In a Chinese study, an incidence of AKI was reported to be 15.4% in patients in the 65-80 age group and 22.2% in the above 80 age group indicating an association with AKI, aging, and worsened outcomes [4]. Older age was associated with higher mortality in a large cohort study with critically ill patients [5]. There is an array of factors which are the cause of a higher incidence of AKI in the elderly from comorbidities that can facilitate AKI, to drugs or surgeries that could act as kidney stressors, to changes in structural and functional aspects of the kidney associated with age-associated [3]. Along with a higher incidence of AKI, the elderly also have decreased repairing and regenerating abilities in response to injury due to factors such as cellular senescence, decreased proliferation, impaired angiogenesis and immunosenescence/inflammaging [6]. Age has also been shown to cause an aggravated response in mice with folic acid-induced AKI [91].

Rhabdomyolysis (RM) is responsible for approximately 10% of AKI cases. RM occurs due to a variety of different factors, with crush injury and excessive drug intake being the main factors [8]. RM leads to damage in the skeletal muscle leading to a release of muscle components such as myoglobin into the circulatory system furthermore leading to AKI and even death [9]. 80% of individuals experiencing a RM event have been reported to progress towards RM-AKI and it has been shown to have a significant impact on short- and long-term prognosis [10]. In the case of RM, falls are responsible for about 56.9% of cases when compared with other causes for the disease and this is of particular interest as 34% of the elderly have been known to experience a fall at least once a year [39]. Multiple clinical reports have also brought to the forefront the effect of sex and age on AKI [12, 13]. Preclinical studies have shown females to be more resistant to AKI when compared with males [14, 15, 16].

Because of the high incidence of AKI in the elderly and because of the gender differences in RM-AKI, the present study was designed to investigate the effect of aging and sex in a mouse model of RM-AKI.

MATERIALS AND METHODS

Animal Models

Male and Female C57BL/6J mice were purchased from Janvier (Le Genest Saint Isle, France). Male mice of the age groups 8 weeks and 26 months were purchased. Female mice of the age groups 8 weeks, 25 months and 26 months were purchased. The mice were maintained in a pathogen-free, temperature-controlled environment where lighting (12-hour light/dark photocycle) was maintained. Ad libitum access to food and water was given to the mice. Anesthetizations of mice were performed before procedures with isoflurane 2-3% v/v. RM was induced by intramuscular injection of 7.5ml/kg of 50% glycerol (99.5% m/v AnalaR Normapur; VWR International S.A/S., Fontenay sous Bois, France) diluted in saline or injected with saline alone as a control [17]. Tail vein blood was collected every week. Mice sacrifices were performed either 2 days or 37 days following administration of 50% glycerol and blood and kidney samples were collected. Experiments were conducted per the Guide for the care and use of laboratory animals of the National Institute of Health, eighth edition and the French Institute of Health guidelines for the care and use of laboratory animals. The project was approved by the local (Inserm/UPS US006 CREFRE) and national ethics committee for animal experiments, CEEA122; Toulouse, France; approval #202012041500456 and 202106301003355.

Biochemical Analysis of Renal Function

Mouse tail vein blood was collected in a microvette. Plasma was obtained through centrifugation of blood at 2000 rpm for 5 minutes at 4°C and the supernatant was collected. Creatinine phosphokinase (CK) and blood urea nitrogen (BUN) concentrations were quantified on a Pentra 400 analyser (Horiba Medical, Grabels, France).

Flow cytometry analysis

The kidneys were rinsed with PBS and digestion was performed as previously reported [18]. Resuspension of the cell suspension was performed with PBS and viability dye (Viability 405/452 Fixable Dye, Miltenyi Biotec, Bergisch Gladbach, Germany) and incubated with Fc block (Miltenyi Biotec, Bergisch Gladbach, Germany) and then incubated with antibodies CD45 PE-Vio 770 REA737 Miltenyi Biotec, CD11b PE-Vio615 REA592 Miltenyi Biotec, F4/80 APC REA126 Miltenyi Biotec, CD3 PE REA641 Miltenyi Biotec, CD4 APC Vio 770 REA604 Miltenyi Biotec, CD8a VioGreen 520 REA601 Miltenyi Biotec, CD19 VioBright

FITC REA749 Miltenyi Biotec, Ly6C PerCP Vio 700 REA796 Miltenyi Biotec (Miltenyi Biotec, Bergisch Gladbach, Germany) and Ly6G Alexa Fluor 700 1A8 Biolegend (Biolegend, San Diego, California, United States of America).

Flow cytometry acquisition was performed on Cytek Aurora spectral flow cytometer (Cytek Biosciences, Fremont, California, United States of America). Files were saved in FCS format and analysed using Cytek Aurora Software (Cytek Biosciences, Fremont, California, United States of America) and FlowJo software (Becton Dickinson, Franklin Lakes, NJ, United States of America).

qPCR

Transcriptional analysis was performed using mouse kidneys by isolation of total RNA using RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) and reverse transcription was performed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, Massachusetts, United States of America). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the One Green qPCR premix (Ozyme, France). The expression levels of HPRT1 were used to normalize the relative expression. The primer sequences used for PCR are shown in the table below.

Serial number	Gene	Forward Primer	Reverse Primer
1	Mouse <i>HPRT1</i>	GGGGCTGTACTGCTTAACCAG	TCAGTCAACGGGGGACATAAA
2	Mouse <i>KIMI</i>	TAGAGACACGGAAGGCAACC	TGCTGCTACTGCTCCTTG TG
3	Mouse <i>CCL2</i>	TTAAAAACCTGGATCGGAACCA	TTAAAAACCTGGATCGGAACCA
4	Mouse <i>NGAL</i>	TCCTCAGGTACAGAGCTACAA	GCTCCTTGGTTCTTCCATACA
5	Mouse <i>H-ferritin</i>	ACTGCACAAACTGGCTACTG	CGTGGTCACCCAGTTCTTTAAT

Table 1: Primers used for real-time PCR

Histological Analysis

From the murine model of rhabdomyolysis, kidneys were fixed in Carnoy or Paraformaldehyde (PFA) solution. Standard histology and immunohistological staining were performed. 4 µm paraffin-embedded tissue sections were cut for immunohistochemistry. The slides were stained with Masson's trichrome to collagen. Nanozoomer 2.0 RS (Hamamatsu Photonics SARL<

Massy, France) was used to scan the sections and ImageJ software was used for staining analysis. The kidney interstitial fibrosis level was measured by calculating the percentage area of collagen staining.

Statistical Analyses

Statistical analysis was done with GraphPad Prism software, (San Diego, California, USA). Statistical analyses between two data sets were performed using unpaired t-tests (Mann-Whitney test) while comparisons between more than two datasets were performed using one-way ANOVA tests. In all cases, $p < 0.05$ was considered as statistically significant.

RESULTS

Effect of aging on RM-AKI

To compare the effect of RM-AKI injury between sexes and age groups, RM was induced in young (2 months old) and old (26 months old) male and female C57BL/6J mice. All four groups were analysed at different time points up to 37 days after glycerol injection (Figure 1A). Blood urea nitrogen (BUN) was measured to evaluate the alteration of kidney function, and plasma creatinine phosphokinase (CK) was measured to evaluate the intensity of muscle damage. These parameters were monitored at various time points up to 37 days after glycerol injection. In all four groups, BUN increased with time, peaking at 48h, and slowly decreasing over time thereafter (Figure 1B and C) and plasma CK showcased a peak at 6h (Figure 1E) followed by a rapid return to basal levels after 1 day.

In young mice, BUN (expressed as AUC) was significantly higher ($p < 0.0001$) in males compared with females (Figure 1D). In parallel, CK at 6h was also significantly higher ($p = 0.046$) in young males compared to young females (Figure 1E). These data indicated the existence of sexual dimorphism in RM-AKI at a young age resulting from a difference in sensitivity in glycerol-induced muscle damage between sexes.

In females, BUN (AUC) was significantly higher ($p = 0.0051$) in old compared to young mice (Figure 1D) with no significant difference observed in the induction of CK at 6h (Figure 1E). In parallel, the BUN to CK ratio was also significantly higher in old ($p = 0.0319$) compared to young females (Figure 1F). In contrast, BUN (AUC) was not significantly different between young and old males ($p > 0.9999$) (Figure 1D) despite a significant reduction of CK at 6h (Figure

1E). In parallel, the BUN to CK ratio was not significantly different between young and old males ($p=0.3480$) (Figure 1F). These data indicated that age increases the sensitivity to RM-AKI specifically in females.

These data indicated that conversely to males, females are resistant to RM-AKI at a young age, and this is unrelated to a change in the sensitivity to glycerol-induced muscle damage. These data also indicated an increased sensitivity to RM-AKI with age in females not in males revealing the existence of sexual dimorphism in the influence of age on RM-AKI sensitivity. Moreover, females turn out to be better suited than males as a model to study the influence of age on RM-AKI.

Effect of age on AKI and fibrosis markers in female mice

AKI is known to cause an increase in the expression of several genes in the kidney such as *Kim1* (kidney injury marker 1), *Ngal* (neutrophil gelatinase-associated lipocalin), *Ccl2* (chemokine (C-C motif) ligand-2), and a decrease in *Fth1* (H-ferritin) [19, 20, 21, 22]. The expression of these genes was measured by qPCR in the kidneys of young and old females 37 days after glycerol injection. RM-induced up-regulation of KIM-1, NGAL-old and CCL-2 mRNAs and down-regulation of H-ferritin were significantly enhanced in old compared to young females (Figure 2 A). These results allowed us to objectivate the increased susceptibility of females to RM-AKI with age at the gene level.

Kidney fibrosis was quantified through the Masson trichrome of collagen (blue stain) 37 days after glycerol injection. As shown in Figure 2B, collagen deposition was significantly increased with RM compared to control in old females but not in young females. These results showed that, in females, the higher susceptibility to RM-AKI with age has a higher deleterious impact on kidney structure.

Effect of age on RM-AKI-induced alterations of kidney immune cells in female mice.

RM-AKI is known to alter the immune cell populations in the kidney with a shift from $F4/80^{\text{high}}CD11b^{\text{low}}$ (R2) to $F4/80^{\text{low}}CD11b^{\text{high}}$ (R1/R0) mononuclear phagocytic cells (MPCs) [18]. To assess whether the increased susceptibility of females to RM-AKI with age could be associated with further alterations of the immune cell populations, flow cytometry analyses were performed on kidney immune cells isolated from young and old females 2 days and 37 days after glycerol injection. Two different time points were selected to observe the differences in the immune cell populations (innate and adaptive) over time. We observed a trend towards

an increase of CD45 (Figure 3A), and a trend towards a decrease of the R2/R1 ratio (Figure 3B), and of CD19 (Figure 3D) at both 2 and 37 days. We also noticed opposite trends towards variation of the CD4/CD8 ratio between 2 and 37 days (Figure 3C). However, none of these changes appeared to be statistically significant.

These results indicate that while some variation trends were observed, it was not possible to objectify significant changes in the immune cell populations associated with RM-AKI in females.

DISCUSSION

Our study investigates for the first time the effect of ageing in RM-AKI considering sex differences.

We found that young males are much more sensitive to RM-AKI than young females. This observation is in agreement with previous reports on mice and rats showing that females are more resistant to acute and chronic injuries, possibly due to sex hormones [23, 24]. Testosterone has been showcased to be responsible for the enhanced susceptibility of males to ischemia-reperfusion injury-based AKI. Female mice are more resistant to ischemia-reperfusion AKI and estrogen administration is responsible for affecting the difference in susceptibility [25]. Similarly, a previous report from our lab showcased the protective effect of estrogen and the effect of estradiol in counteracting against AKI [26]. Sirtuin 6 has been shown to play an important role in susceptibility to AKI, especially in males [27]. Similarly, females are more resilient to ferroptosis-induced AKI where NRF2 would be a cause for the resilience and preclinical study revealed the possibility of targeting NRF2 for ferroptosis sensitivity [28].

Our most original observation is that, in contrast to males, females increase their sensitivity to RM-AKI with age, revealing the existence of sexual dimorphism in the influence of age on RM-AKI sensitivity. Preclinical studies illustrating the effect of gender and age on RM-AKI are limited. Previous reports show that aged mice were shown to have an aggravated effect of AKI due to folic acid-induced AKI when tested in male mice showing early age-associated changes [91]. This report showcased that aged kidneys showcased a switch to proinflammatory cell death and lost local nephroprotective factors. This could be an indication of the following aspects that different types of AKI could have a different effect on sensitivity to AKI due to the sex and age of the mice as well.

Aged mice with bilateral ischemia-reperfusion AKI were shown to have an increase in proinflammatory cytokines indicating an association with inflammaging and had a significant increase in renal fibrosis due to persistent M1 inflammation and prolonged G1 cell cycle arrest [29]. Interestingly, T cells were shown to be highly affected in cisplatin-induced AKI in aged mice [30]. The effect of kidney and inflammation markers was observed, and upregulation of prominent kidney and inflammation-associated markers such as KIM-1, NGAL and CCL2 was observed. Fibrosis and kidney structure damage were observed to be more protuberant in old females with RM-AKI in comparison with younger females. A trend was observed in the immune cell populations for CD45, R2/R1, CD4/CD8 and CD19 but it was not statistically significant. Although we cannot exclude that the absence of significance is due to the limited group mice size, our observation suggests that the female-specific increased sensitivity to RM-AKI is unrelated to inflammation. This could be indicative of the fact that the inflammation markers and immune cells currently tested may not give sufficient details on the effect of ageing on RM-AKI in females, and complementary studies will be required to uncover the responsible factors. One approach will be to conduct transcriptomic analysis to identify the genes and pathways responsible for disease progression in RM-AKI specifically in females.

Clinical studies illustrated the higher incidence of severe acute renal failure in males in comparison with females and the evidence of age being an important factor where those >65 years old had a higher risk [31]. Men are more likely to develop incident end-stage renal disease (ESRD), while women have a higher incidence of chronic kidney disease (CKD) [32, 33]. A clinical study of RM-AKI has shown the incidence to be lower in females in comparison with males and where the median age was observed to be 49 [10]. Sex hormones have been known to influence sexual dimorphism leading to a downstream effect on the pathogenesis of AKI and CKD [34]. Clinical studies have shown that females have more glomeruli per gram of kidney weight, lower absolute glomerular filtration rate, higher renovascular resistance and lower renal plasma flow [35]. Ischemia-reperfusion injury studies have shown females to have a reno-protective profile because of heat shock protein HSP72 [36]. Similarly, studies have shown how sex has a different response to different types of AKI, for example, while female mice have been known to have higher susceptibility towards cisplatin-induced AKI, they are protected from ischemia-reperfusion AKI [37]. This leads to the hypothesis that sex can lead to different responses to AKI treatment which has been revealed in treatment with erythropoietin where male mice are protected from cisplatin-induced AKI while female mice showcased no such protective response [38, 39].

In conclusion, the results of the present study suggest that females could be a better model for understanding the effects of ageing on RM-AKI due to their sensitivity towards the disease. Further studies are required to address the underlying mechanism of age-related differences in females by looking at multiple age groups including young, adult, elderly and frail and the effect due to RM-AKI as well as sex and aging-associated markers to assess the specific genes responsible for disease progression and to further assess the possibility of analysing old females as a suitable model to study pharmaceutical candidates to prevent AKI.

LIMITATIONS

Further studies need to be conducted with a larger sample size to confirm the results obtained due to the limited sample size of old females in the current study.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

S.N. Rao performed RM-AKI experiments, performed the data analysis, and wrote the initial version of the manuscript. S. Faguer revised the manuscript, and A Casemayou, M. Buleon and G. Feuillet performed RM-AKI experiments. E Riant and A Zakaroff-Girard performed cell sorting. J. Belliere, J. P. Schanstra and J.S. Saulnier-Blache designed the study and oversaw overall direction, planning and manuscript writing.

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FIGURES LEGEND

Figure 1: Effect of gender and age differences on RM-AKI

A: Schematic diagram representing experimental procedure where mice were injected with 50% glycerol for induction of rhabdomyolysis (RM-AKI) in six different groups young (2 months old) and old (26 months old) for males and females respectively as well as young (2 months old) and old (25 months old).

B: Kinetics of young males and old males based on Blood urea nitrogen (BUN).

C: Kinetics of young females and old females based on Blood urea nitrogen (BUN).

D: Dot plot indicating the area under the curve from each of the groups based on blood urea nitrogen (BUN)

N=6 for young males, N=6 for old males, N=6 for young females and N=4 for old females Stat = Brown-Forsythe and Welch ANOVA test

E: Dot plot indicating creatinine phosphokinase (CK) at 6 hours post rhabdomyolysis induction
N=6 for young males, N=6 for old males, N=6 for young females and N=5 for old females Stat = Brown-Forsythe and Welch ANOVA test

F: Dot plot indicating the ratio of blood urea nitrogen (BUN) at 48 hours post rhabdomyolysis induction to creatinine phosphokinase (CK) – hours post rhabdomyolysis induction.

N=6 for young males, N=6 for old males, N=6 for young females and N=4 for old females Stat = Brown-Forsythe and Welch ANOVA test

Figure 2: Effect of age on kidney markers and fibrosis in RM-AKI I females

A: Dot plots of qPCR analysis comparing expression in young and old females with RM-AKI

B: Histological analysis through Masson staining for young versus old females in Control and RM-AKI

N=2 for the control young female, N=4 for young females with RM-AKI, N=2 for old female control and N=3 for old females with RM-AKI; Stat = Brown-Forsythe and Welch ANOVA test

C: Zoom in of Masson staining of young versus old females in control and RM-AKI

Figure 3: Flow cytometry analysis of kidney immune cells in young and old females subjected to RM-AKI at 2 and 37 days

A: Illustrative example of CD45 populations obtained by flow cytometry. Contour plot of CD45 vs. Live Dead obtained from digested kidneys. Contour plots and statistics were generated using FlowJo.

N=4 for young females 2 days after RM induction, N= 4 for old females 2 days after RM induction, N=3 for young females 37 days after RM induction and N=3 for old females 37 days after RM induction; Stat= Mann-Whitney test

B: Contour plot of F4/80 vs CD11b gated on CD45 live cells obtained from digested kidneys. F4/80^{low}CD11b^{high}cells (R1) were dominant MPC in the RM-AKI condition. Contour plots and statistics were generated using FlowJo.

N=4 for young females 2 days after RM induction, N= 4 for old females 2 days after RM induction, N=3 for young females 37 days after RM induction and N=3 for old females 37 days after RM induction; Stat= Mann-Whitney test

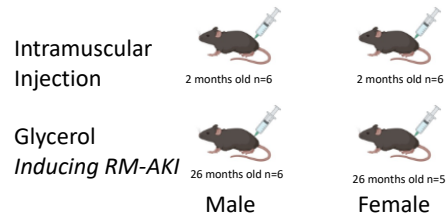
C: Illustrative example of T cells obtained by flow cytometry. Contour plot of CD8 vs. CD4 gated on CD45 live cells obtained from digested kidneys. Contour plots and statistics were generated using FlowJo.

N=4 for young females 2 days after RM induction, N= 4 for old females 2 days after RM induction, N=3 for young females 37 days after RM induction and N=3 for old females 37 days after RM induction; Stat= Mann-Whitney test

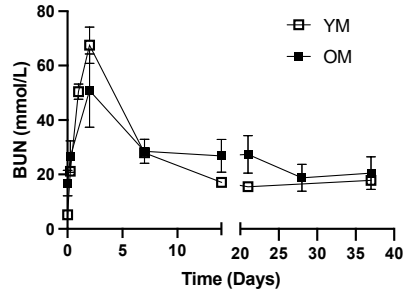
D: Illustrative example of B cells obtained by flow cytometry. Contour plot of CD19 vs. CD3 gated on CD45 live cells obtained from digested kidneys. Contour plots and statistics were generated using FlowJo.

N=4 for young females 2 days after RM induction, N= 4 for old females 2 days after RM induction, N=3 for young females 37 days after RM induction and N=3 for old females 37 days after RM induction; Stat= Mann-Whitney test

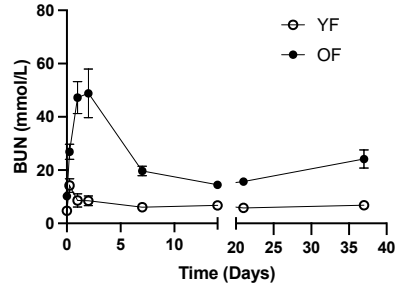
A



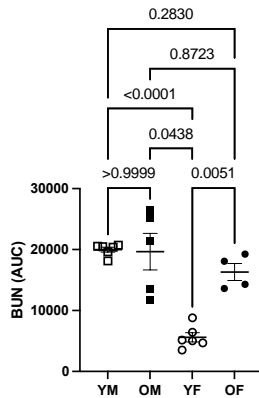
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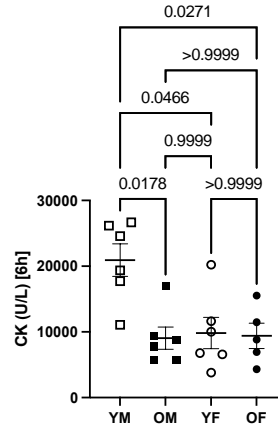
C



D



E



F

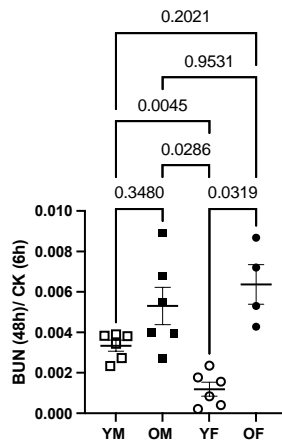


Figure 1

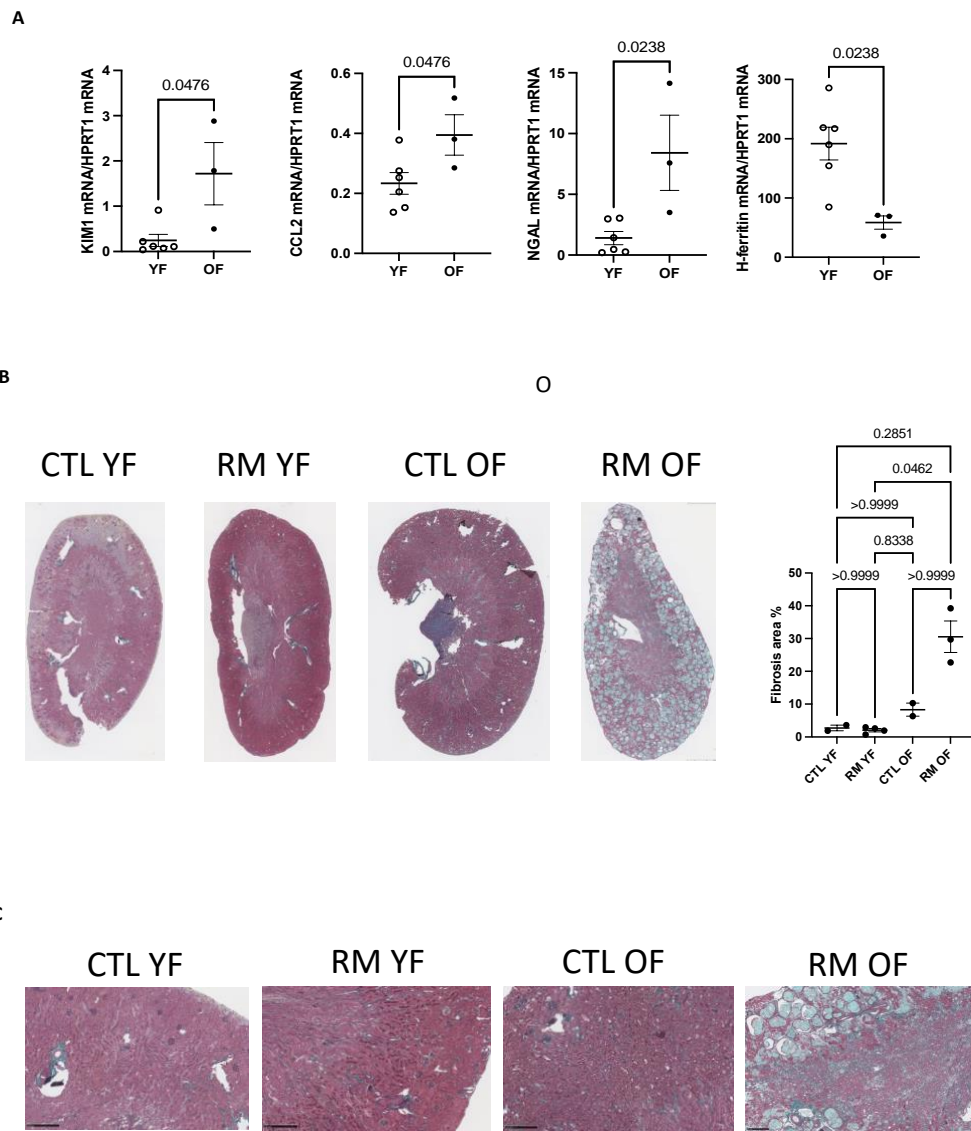


Figure 2

A

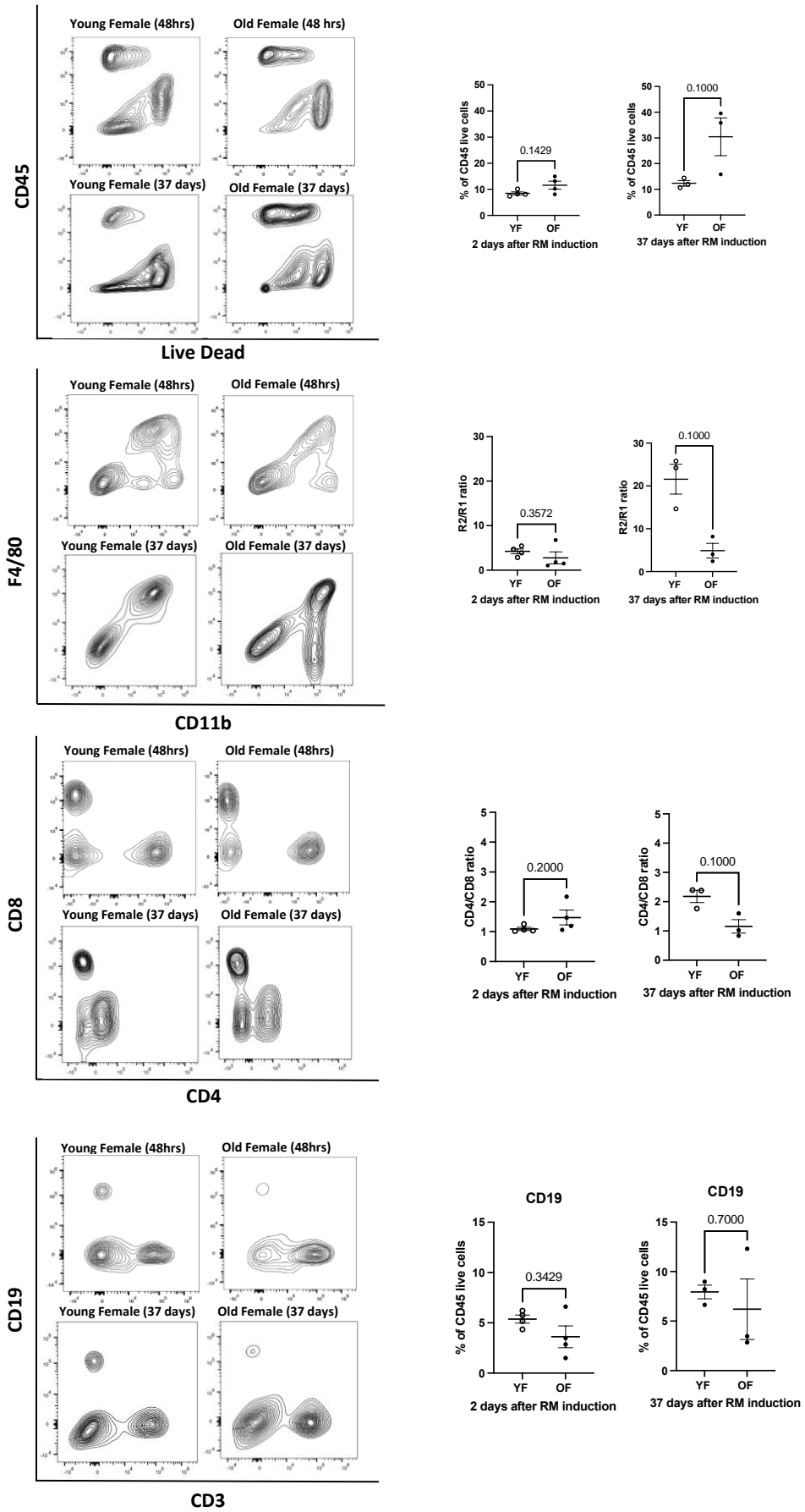


Figure 3

GENERAL DISCUSSION AND PERSPECTIVES

RM-AKI is a disease that has been well studied and the importance and role of MPCs in the disease progression has been well reported. Current studies on immune cells and specifically on the effect of MPCs on the disease have had their limitations due to the use of traditional flow cytometry and a limited gene set giving information on only one aspect of the disease, this work highlighted the importance of a high throughput technique to assess the immune landscape and the factors responsible for the disease progression.

We performed scRNAseq of C57BL/6J mice with and with RM-AKI to illustrate the conversion from control to RM-AKI condition. Immune cell clusters for macrophages, monocytes, dendritic cells, neutrophils, natural killer T cells, B cells and T cells were obtained. A relative increase in the proportion of macrophages, monocytes and neutrophils was observed in RM-AKI. Interestingly, adaptive immune cells (T and B cells) were present in both conditions, but no particular polarization was observed at such an early stage of the immune response. This could be because 2 days after RM induction may not be sufficient to induce a specific adaptive immune response. Functional enrichment analysis showcased pathways associated with B cells such as the B cell receptor signalling pathway and for antigen processing and presentation while for T cells pathways associated with Th1, Th2 and Th17 cell differentiation were observed. *Socs3* was observed to be the upregulated gene expressed in both B cells and T cells which could indicate an inflammatory nature [84]. Future studies where scRNASeq could be performed 7 days after RM induction which will give light to the determination of adaptive immune cell changes due to B cells and T cells showcasing a later stage of immune response. The limitation due to the low number of cells could also be another cause for the limited array of information available for the adaptive immune cells.

Previous studies [53] showcased the importance of macrophages, monocytes and specifically the MPC in RM-AKI. It was of interest to zoom into the MPC clusters to obtain the differences between RM-AKI and control conditions. An augmentation of macrophage and monocyte population was observed in the RM-AKI condition both qualitatively and quantitatively. A decline in the dendritic cell population was observed in the RM-AKI condition which was observed. Sub-clustering of the MPC populations showcased populations specific for macrophages, monocytes, and dendritic cells. Qualitative and quantitative analysis revealed

clusters specific to control and RM-AKI condition and of specific interest a cluster present in both conditions but augmented in RM-AKI. Classification with canonical M1 and M2 polarization markers showcased its limitation in the classification of the clusters due to its heterogeneous expression. MPC subclusters were successfully categorized based on previous classifications [53]. Furthermore, our data was compared with a renal-ischemia reperfusion model which showcased that our subclusters had either resident or infiltrative profiles [85].

The unique cluster possibly acting as a transition cluster between control and RM-AKI condition was studied through trajectory analysis and differential expression brought to light MHCII class genes as the top downregulated genes. To expression of these genes was assessed in all the MPC subclusters where a high expression was observed in control clusters while low expression was observed in RM-AKI clusters. The unique cluster showcased bimodal expression of MHCII markers indicating the possibility of this cluster acting as a transitory cluster from control to RM-AKI condition. We hypothesize that this unique cluster has a role as a transitory cluster from MHCII^{high} cells in homeostatic conditions which reprogram into MHCII^{low} cells due to injury. Previous studies have showcased how after renal ischemia-reperfusion, MHCII downregulation has been associated with transcriptional reprogramming of resident MPC towards a developmental state [86]. Since we have a hypothesis of the transitory nature of cluster 8, it would be of interest to look at the cell fate transition through modelling cell fate dynamics giving probable information of initial, intermediate and terminal states which would give information on the accuracy of our hypothesis in differentiation of cells from one state (control) to another (RM-AKI disease) [87]. Pathway analysis revealed “Cell cycle” and “cellular senescence” to be highly expressed in the unique cluster. It would be of great interest to perform RNA velocity, cell fate modelling and further pathway enrichment studies to be able to study the transition in this unique cluster from control to RM-AKI state. Furthermore, studies specifically looking at this cluster in future studies with scRNA-seq studies for RM-AKI at different time points and age groups would give more information on the impact of this transition cluster in disease progression over time and age groups. Single-cell spatial transcriptomics could be performed to assess the spatial localization of this unique cluster which was not possible in the current study due to limitations due to the small cluster size for analysis through scRNASeq.

As cellular senescence was observed to be a pathway of interest in the MPC in RM-AKI, we were interested in asserting if there is any similarity in the signature between MPC and TECs.

Tubular cells have been known to have a role in senescence due to their role in the accumulation of senescent cells after kidney injury and further in kidney damage progression [68,88]. KEGG analysis of public single-cell data showcased no specific comparable enriched genes between our unique cluster and TECs. It is interesting to perform future studies of RM-AKI where TECs are also analysed to illuminate the variations due to the disease.

Cellular senescence and upregulation of ageing-associated genes *p21* and *Fgf2* brought to light the possibility of the use of senolytics as a treatment strategy for RM-AKI. Dasatinib and quercetin which is known to reduce senescent cells and have an impact on renal fibrosis in cisplatin-induced murine AKI and unilateral renal ischemia-reperfusion [89]. A combination treatment of DQ with pre-treatment and treatment after RM induction was performed which showcased a reduction in BUN, attenuation in injury-associated markers and a reversion in the MPC phenotype. Studies at a later time point would probably give more information on the effect of adaptive immune cells. A scRNA-Seq study could give information on the effect of senolytics on our unique cluster and whether our hypothesis of its role as a transition cluster can be confirmed. Since older populations have higher senescent cells, it would be interesting to perform DQ treatment on old mice with RM-AKI to see if it would have a specific effect on RM-AKI.

This second part of this thesis highlighted the effect of aging in response to RM-AKI and the differences in response to RM-AKI due to sex. We demonstrated the association between age increasing the sensitivity to RM-AKI and brought to light that females are better suited for the study of the effect of age on RM-AKI. Previous studies have uncovered that female mice are more resistant to ischemia-reperfusion AKI where estrogen administration was shown to be a causal factor in the varying response between sexes [90]. The effect of estrogen on AKI was shown to be protective while estradiol was shown to have counteracting effects that have further been demonstrated in AKI in previous studies [90] and age has been presented as an important factor causing an aggravated response to folic-acid AKI [91]. Further studies are necessary comparing RM-AKI-affected mice with control mice to assess the differences due to sex and age. Studies assessing the effect of gender-associated markers on RM-AKI would give detailed information on the association between gender and disease.

An upregulation of certain kidney-associated markers indicated the susceptibility of females to RM-AKI with age and fibrosis studies indicated the effect to be more prominent in old mice

when compared with young mice. Future experiments need to be performed which would assess the genes specifically responsible for higher susceptibility due to ageing would be beneficial. It would be interesting to look at the ageing-associated markers. This would give an outlook on the factors responsible for increased susceptibility to RM-AKI because of age.

To unravel the susceptibility of females to RM-AKI due to age, it is of interest to analyse the immune cell populations obtained between the different age groups and time points. Flow cytometry was performed on kidney immune cells obtained from young and old female mice 2 days and 37 days after RM induction. A variation in immune cell populations was observed but it was not observed to be statistically significant. Further studies comparing the control and RM-AKI populations would give more details on the effect due to the disease and age through the study of a larger fraction of immune cell populations. It would be of vital interest to compare control and RM-AKI-affected mice in multiple age groups young, adult, elderly, and frail to be able to understand the effect of ageing on the disease and to look at the ageing-associated markers and see their effect on the disease to be able to explain if the ageing associated pathways have any effect on the disease. Similarly, it would be of vital importance to perform transcriptome studies to assess the exact genes and pathways responsible for the susceptibility of females to RM-AKI with age as it may not be associated with inflammation and the current studies of immune cell populations. RNASeq is currently in the process of assessing the transcriptome studies where a preliminary study using the murine microenvironment cell population counter method would be used to identify the different immune cell types through RNASeq [92].

CONCLUSION

Through this work, we were able to identify the immune landscape responsible for the transition from control to RM-AKI condition through scRNASeq. A closer look into the MPC subclusters led to the classification based on a previous publication [53] as classification based on traditional M1 and M2 polarization was observed to be ineffective due to the heterogeneous nature of expression.

The most important observation was the identification of a unique subcluster in MPC showcasing a bimodal expression of MHCII^{high} and MHCII^{low} cells, leading to our hypothesis that this cluster could act as a transitory cluster allowing reprogramming from the control to RM-AKI condition. We further identified the possibility of senolytics as a nephroprotective agent for the treatment of RM-AKI.

An interesting aspect of the study was analysing the effect of aging concerning sex differences where we observed increased sensitivity to RM-AKI with age in females leading to our hypothesis of females as a probability as a better model system for the study of aging in RM-AKI. Additional work would be necessary to confirm this hypothesis.

Following the present work, a few different research avenues would be interesting to explore. First, not enough information is available on the cause of the increased sensitivity in females with age. It would be of utmost interest to perform spatial transcriptomics studies comparing different age groups in males and females. This would give an outlook on the exact factors responsible for the increased sensitivity which could then bring to light possible pharmacological targets. Secondly, although we have specified the identification of a unique cluster with a role as a transitory role, the importance of this cluster must be further detailed. Studies specific to this cluster through scRNASeq could give information on the impact on the cluster after senolytic treatment. This cluster could be purified through GateID cell sorting generating intuitive FACS gates identified through scRNASeq. This cluster could then be under CRISPR screening where the specific genes to the cluster could be knocked out giving an array of information on the role of the genes and pathways on the disease progression. As this cluster has an overexpression of *Stathmin1* it could be of interest to study the effect of knockout of *Stathmin1* as it would give a larger outlook on the impact on this cluster. *Stathmin*-deficient mice have been shown to have an impaired tubular repair process as well as in recovery after ischemia-reperfusion acute kidney injury [93]. Long-term studies delving into the effect of the absence of *stathmin* on acute kidney injury need to be performed to be able to explain the

prolonged effect in the worsening of kidney function and the possibility of the development of permanent and chronic renal injury. Thirdly, although we have looked at the immune cell populations in control and RM-AKI condition we have only looked 2 days after RM-AKI induction which may not give a full picture of the effect of RM-AKI long term. It would be interesting to study the cells observed at a much later stage of RM-AKI through scRNASeq giving a highlight to adaptive immune cells. A study with multiple rounds of exposure to RM would correlate with what is observed in human patients and this may provide correlation with clinical results. Finally, since AKI has been known to progress towards CKD it would be vital to understand the progression towards CKD. This could be done with a RM-AKI model and the progression towards CKD could be studied with spatial transcriptomics. This would probably give better information on the therapeutic avenues aimed at preventing the transition from AKI to CKD. It would be vital to understand if senolytics can be a therapeutic strategy for different types of AKI and the long-term effects of senolytics treatment in AKI need to be studied.

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