THE ROLE OF RETINOIC ACID SIGNALING IN ACUTE KIDNEY INJURY

By

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Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Cell and Developmental Biology

August 2015

Nashville, Tennessee

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To Norie,

Thank you

Acknowledgements

The work presented in this dissertation was performed in the Department of Cell and Developmental Biology, Vanderbilt University School of Medicine during the years of 2010-2015. I would like to thank the Department for their continued support during this time and providing such a diverse scientific environment during my years. I would also like to thank the Center for Kidney Disease in the Division of Nephrology for providing such an exciting and collaborative environment for my research. I would also thank the Program in Developmental Biology for their support and strong commitment to the training of developmental and regenerative biology.

I would first like to thank my mentor, Mark de Caestecker, who has been an instrumental part of my scientific development. He has "adopted" me and provided me an opportunity to restart my graduate career. He taught me perseverance and the intellectual flexibility how to correct the direction of a project when I encounter unexpected results. Without his enthusiasm, guidance, and daily new ideas this project would never have been the same. I would also like to thank the other past and present members of the de Caestecker lab, Kevin Abney, Arunima Datta, Andrea Frump, Sampa Ghose, Mark Jones, Johnathan Lowery, Tatiana Novitskya, Allison Prewitt, Radostin Penchev, Nataliya Skrypnyk, and Ke Xin Zhang.

Of course none of this work would have come to fruition without the collaborations, technical support, reagents, and advice of many people including Eric de Groh, Volker Haase, Neil Hukriede, Cathy Mendelsohn, and Lauren Skvarca. I would also like to include extreme gratitude to my previous mentors and bosses in science including Haruko Kazama, Nori Satoh, and William Jeffery.

To my amazing fiancée, Norie. It's no secret to anyone who knows us that there is little change I would have gotten to this point without you. My life has been different since the day we met, and I'll never know how to thank you for your support over the past four years.

I am forever appreciative of my friends who have been with my through this part of the journey in science and life.

I would especially like to thank my committee, David Bader, Scott Baldwin, Guoqiang Gu, and Ray Harris for keeping me on track and providing me with invaluable advice. I'll never forget a committee meeting in 2012 where the members suggest focusing on a part of my project going. That was the moment that my thesis begin to take shape.

Finally, this would not be possible without all the support of my family inside and outside of the country. They've been there from the start of my endeavors in science and allowed me to pursue my journey.

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List of Abbreviations

ALDH	aldehyde dehydrogenase
α-SMA	alpha smooth muscle actin
ANOVA	analysis of variance
AKI	acute kidney injury
AQP1	aquaporin1
Arg1	arginase1
ATRA	all trans retinoic acid
β-Gal	beta-galactosidase
CCL	CC chemokine ligands
CD	collecting duct
Col1a1	collagen, type I, alpha 1
CSF1	colony stimulating factor1
CX3CL1	(C-X3-C motif) ligand 1
Cyp26b1	cytochrome P450 26B1
DAB	3,3'-Diaminobenzidine
DNRAR	dominat negative retinoic acid receptor
FACS	fluorescently activated cell sorting
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
GM-CSF	granulocyte-monocyte-colony stimulating factor
H&E	hematoxylin and eosin
HPF	high power field
HRP	horseradish peroxidase
hsp68	heat shock protein 68
iNOS	inducible nitric oxide synthase
IL	interleukin
IR-AKI	ischemia-reperfusion induced acute kidney injury
Kim1	kidney injury molecule-1
LC	liposomal clodronate
LPF	low power field
LTL	lotus tetragonolobus lectin
LV	liposomal vehicle
Ly6G	lymphocyte antigen 6 complex, locus G
MACS	magnetically activated cell sorting
MGL1	macrophage galactose-type lectin-1
MPO	myeloperoxidase
MR	mannose receptor
OCT	optimal cutting temperature
OM	outer medulla
PBS	phosphate buffered saline
PEPCK	phosphoenolpyruvate carboxykinase

pH3	phosphohistone-H3
PTEC	proximal tubular epithelial cell
qRT-PCR	quantitative real-time polymerase chain reaction
RA	retinoic acid
Raldh	retinaldehyde dehydrogenase
RAR	retinoic acid receptor
RARE	retinoic acid responsive element
Rbp1	retinol binding protein 1
SEM	standard error of the mean
SR	serius red
TGF-β1	transforming growth factor, beta 1
TIS	tubular injury scores
TNFα	tumor necrosis factor alpha
YFP	yellow fluorescent protein

Abstract

Retinoic acid (RA) has been used therapeutically to reduce injury and fibrosis in models of acute kidney injury (AKI), but little is known about whether and how this pathway is normally regulated, and what role it plays in regulating injury and repair after AKI. In these studies we show that RA signaling is activated in mouse and zebrafish models of AKI, and that these responses limit the extent of injury and promote normal repair. These effects are mediated through a novel mechanism by which RA signaling coordinates the dynamic equilibrium of pro-inflammatory M1 spectrum vs. alternatively activated M2 spectrum macrophages. According to this model, direct repression of pro-inflammatory macrophages by locally synthesized RA reduces macrophage-dependent injury post-AKI, while locally synthesized RA activates RA signaling in injured tubular epithelium, which in turn promotes alternatively activated M2 spectrum macrophages. Since RA signaling plays an essential role in kidney development but is repressed in the adult, these findings provide evidence of an embryonic signaling pathway that is reactivated after injury and plays an important role in reducing injury and enhancing repair after AKI.

CHAPTER I

Introduction

This chapter was published under the tile of 'Kidney Regeneration: Lessons from Development' in *Current Pathobiology Reports* in March 2015 (Chiba et al., 2015b).

1. Overview

Acute kidney injury (AKI) is a common disorder resulting from often multi-factorial ischemic, toxic and septic insults to the kidney (Ali et al., 2007; Lameire et al., 2013). However, incomplete repair and abnormal tissue remodeling after injury is associated with progressive fibrosis and renal insufficiency such that patients with otherwise apparently reversible AKI are at increased risk of progressive chronic kidney disease (CKD) and eventually end stage renal disease (Wu et al., 2011; Bucaloiu et al., 2012; Coca et al., 2012; Jones et al., 2012). Despite these sobering clinical data, the mechanisms regulating cellular repair and tissue remodeling after AKI are incompletely understood. Given the lack of progress in developing effective clinical therapeutics to enhance tubular repair after AKI, a major focus of AKI research has been to better understand the cellular types, responses and signaling pathways mediating these repair mechanisms.

Much of the work studying the cellular mechanisms of repair after AKI has been performed using ischemia-reperfusion models of AKI (IR-AKI) in rodents (Bonventre and Yang, 2011). These models provide reproducible and defined temporal progression from injury to repair that facilitates analysis. However, there are clearly species differences in responses to AKI. For example, studies using p53 inhibitors in rat and mouse models of IR-AKI suggest that the inflammatory response plays a dominant role in determining the extent of injury in mice (Sutton et al., 2013), while tubular apoptosis appears to be a more important determinant of renal injury in rats (Kelly et al., 2003). In addition, there is concern that the severity of injury in rodent models of IR-AKI does not reflect the more focal and subtle renal injury in patients with AKI (Rosen and Stillman, 2008). This is compounded by the fact that renal biopsies are rarely performed in patients with AKI (Stillman et al., 2008), so it is difficult to know whether the pathophysiologic mechanisms of injury and repair are similar or distinct. Having said that, limited post-mortem studies indicate that there are shared elements in rodent and human AKI. These include tubular injury in the cortico-medullary region of the kidney, including occasional tubular necrosis and apoptosis, more extensive epithelial sloughing, increased tubular proliferation and the presence of peri-tubular inflammatory infiltrates (Solez et al., 1979; Solez et al., 1993; Takasu et al., 2013). Therefore, while rodent models of IR-AKI have their limitations, I contend that they share the same basic cellular responses to injury as those occurring in human AKI, even though the extent to which these responses contribute to injury and repair after AKI may vary between species.

2. Cellular mechanisms of repair after AKI

A number of genes involved in embryonic kidney development, including Pax2, Lhx1 and components of the Wnt, Notch, Hedgehog and BMP signaling pathways are reactivated in the adult kidney after IR-AKI in rodents (Simon et al., 1999; Terada et al., 2003; Villanueva et al., 2006; Kobayashi et al., 2008; Fabian et al., 2012). This has led to the belief that reactivation of pathways promoting cell proliferation and specification during embryonic development drive similar regenerative responses in the adult kidney after injury (Cirio et al., 2014). However, with few exceptions (notably activation of the BMP signaling pathway), the functional role of these pathways in regenerative repair after AKI has either never been tested, or has been shown to promote dysfunctional repair and fibrosis rather than cellular regeneration. In this chapter I will take a systematic approach to address this problem. First I will discuss what is known and what is debated about the cellular mechanisms of repair after AKI. Then, taking advantage of recently published, cell-specific translational profiling studies that were performed in two mouse models of AKI (Grgic et al., 2014; Liu et al., 2014), I will provide a comprehensive analysis of the expression profiles of genes and pathways involved in embryonic kidney development that are regulated after AKI, and discuss what published data are available to explain the functional roles of these genes and pathways in regenerative repair and tissue remodeling after AKI. I will also discuss that while injury regulates many of the same pathways involved in embryonic development, these are often regulated in different cell types and lack the coordinated balance required for properly organized tissue regeneration and remodeling after injury. These findings have parallels in cancer biology, where mis-expression and/or subversion of the range

of different developmentally regulated pathways by cancer cells (or the host micro-environment) have been the focus of therapeutic developments to target initiation and progression of diverse malignancies (Kiesslich et al., 2012).

Complete resolution of injury is dependent not only on efficient regeneration of damaged tubular epithelium but also on the restoration of tissue oxygenation by damaged microvasculature (Bonventre and Yang, 2011; Sharfuddin and Molitoris, 2011). These responses are determined by the intrinsic ability (or inability) of these cells to regenerate, and by their local microenvironment, in particular the effects of paracrine factors secreted by macrophages and vascular pericytes on tubular repair and vascular stability, respectively (Fligny and Duffield, 2013; Huen and Cantley, 2014). I will discuss the four dominant cell types involved in tissue repair after IR-AKI: renal tubular epithelial cells, endothelial cells, myofibroblasts and vascular pericytes, and macrophages (Bonventre and Yang, 2011; Kramann and Humphreys, 2014). Since each of these cellular compartments has its origins during kidney development, I will discuss the cellular origins and role of these cell types during embryonic development of the kidney, and will discuss how each cell type functions to be part of an integrated cellular response that is required for proper development and for tissue repair after AKI.

Cellular compartments during mammalian kidney development

Tubular epithelial cells are derived from nephron progenitor cells within the cap mesenchyme (CM) (Costantini and Kopan, 2010; Kopan et al., 2014). Coordinated signals derived from ureteric bud (UB) epithelium, which gives rise

the collecting duct (CD) system in the adult kidney, as well as paracrine signals from the surrounding stroma, regulate the survival and coordinated differentiation of CM cells into polarized epithelium of the renal vesicle (RV). As a result of coordinated signaling from within the RV, these structures subsequently undergo expansion and patterning to form proximal and distal nephron segments, fusing with ureteric epithelium at an early point to form the complete nephron. Stromal mesenchyme, which along with the CM is essential for patterning the UB, gives rise to fibroblasts, mesangial cells and vascular pericytes in the adult kidney (Humphreys et al., 2010). Paracrine factors secreted by CD epithelium also induce stromal cells to differentiate into peri-tubular smooth muscle cells (Yu et al., 2002). Endothelial cells in the adult kidney are largely (but probably not entirely) derived from Flk1 positive endothelial progenitor cells within the stromal mesenchyme (Herzlinger and Hurtado, 2014). Patterning of the renal vasculature is not completely understood, but the formation of glomerular capillaries is regulated by epithelium and mesangial cell-derived factors (Takabatake et al., 2009; Haege et al., 2012), while global patterning of renal vasculature is dependent on the stromal mesenchyme itself (Hum et al., 2014). Conversely, tissue oxygenation from vascular perfusion is required for CM differentiation during the last trimester of pregnancy (after 15.5 days post-fertilization in mice) (Rymer et al., 2014). There are no published data on the functional role of macrophages in kidney development. However, macrophages are required for normal breast, bone and brain development as well as correct patterning of the retinal vasculature (Wynn et al., 2013). Moreover, temporal analysis of macrophages in the developing kidney shows that these cells are intimately associated with tubular epithelium from the earliest stages of

kidney development, and that these cells express markers of regenerative or trophic macrophages (Rae et al., 2007). Taken together therefore, while our understanding of the functional role of all four-cell types in kidney development are incomplete, these data establish that there are extensive functional interconnections between epithelial, endothelial, stromal and macrophage lineages during embryonic kidney development.

Cellular compartments involved in tissue repair after AKI

Epithelium

Tubular epithelial cells, particularly cells in proximal tubular segments of the kidney, are the primary targets of injury after IR-AKI (Bonventre and Yang, 2011). Recovery of this damaged epithelium is essential to restore normal renal function, and incomplete renal tubular repair is thought to be responsible for tubular atrophy and interstitial fibrosis that gives rise to progressive CKD in patients post-AKI (Venkatachalam et al., 2010). In IR-AKI, tubular repair, which occurs a short time delay after the initiating injury, is characterized by robust proliferation and subsequent re-differentiation of injured but surviving tubular epithelial cells (Witzgall et al., 1994; Humphreys et al., 2011; Kusaba et al., 2014). Genetic lineage studies indicate that tubular repair does not depend on the migration of extra-tubular stem cells into the regenerating tubule (Humphreys et al., 2008; Kusaba et al., 2014). Moreover, clonal analyses and sequential thymidine analogue pulse-chase experiments, indicate that regenerating cells are uniformly distributed throughout the regenerating proximal tubular epithelium after IR-AKI (Humphreys et al., 2011; Berger et al., 2014; Kusaba et al., 2014;

Rinkevich et al., 2014). This supports the hypothesis that this regenerative response is unlikely to involve the proliferation of sub-set of intra-tubular progenitor or stem cells. However, this issue is still vigorously debated since there is evidence that there is a subset of epithelial cells that express the stem cell markers CD133 and CD24 in adult human kidneys, and that these cells have the capacity to regenerate tubular segments *in vitro* and in orthotopic transplant models (Lazzeri et al., 2007; Angelotti et al., 2012). However, regenerating tubular cells express *CD133*, *CD24*, the tubular injury marker *Kim1*, and the de-differentiation marker, *vimentin* mRNAs after IR-AKI in mice (Kusaba et al., 2014), and CD24 positive cells have also been shown to express Kim1 in human kidneys (Smeets et al., 2013). This has led to the alternate hypothesis that CD24 and CD133 expression reflects de-differentiation of injured cells rather than a stem cell state (Berger et al., 2014; Kusaba and Humphreys, 2014a; Kusaba and Humphreys, 2014b). Having said that, long-term lineage pulse-chase experiments indicate that tubular cells have a remarkable regenerative capacity after IR-AKI (Rinkevich et al., 2014). This raises the possibility that expression of these markers reflects an acquired phenotypic change from an adult, terminally differentiated epithelium to a progenitor-like state with greater proliferative capacity, more closely resembling the embryonic kidney epithelium.

Endothelium

Endothelial injury increases the extent of injury after IR-AKI by further decreasing capillary blood flow and increasing inflammatory cell recruitment (Sharfuddin and Molitoris, 2011). Importantly, there is increasing evidence that peri-tubular capillary rarefaction that occurs after IR-AKI (Basile et al., 2001;

Horbelt et al., 2007), promotes long-term tissue hypoxia, which impairs tubular repair and resolution of fibrosis (Tanaka et al., 2014). The mechanism of microvascular rarefaction is unclear since there is little evidence of endothelial cell death or proliferative repair after AKI (Horbelt et al., 2007; Basile et al., 2011). However, bi-directional signaling between vascular pericytes and endothelium regulates vascular stability, suggesting that capillary rarefaction might result from loss of normal pericytes-endothelial interactions after AKI (Schrimpf et al., 2014). Support for this hypothesis comes from a series of studies demonstrating that interference with pericytes-derived signals that stabilize (TIMP3 and EphrinB2) or destabilize (VEGF and ADAMTS1) the microvasculature exacerbate or attenuate respectively, renal fibrosis after AKI (Lin et al., 2011; Schrimpf et al., 2012; Kida et al., 2013).

Fibroblasts and pericytes

Wound healing studies indicate that expansion of collagen producing myofibroblasts plays an important role in tissue remodeling, including epithelial repair and vascularization (Hinz et al., 2007). However, persistent expansion of myofibroblasts is associated with fibrosis. In the kidney, it is likely that myofibroblasts are derived from different cell types (LeBleu et al., 2013). However, fate mapping using FOXD1 Cre mice to label stromal lineages during embryonic kidney development, indicates that the bulk of these cells originate from vascular pericytes that have delaminated from the vessel wall post-AKI (Humphreys et al., 2010). Thus expansion of myofibroblasts is intimately linked with microvascular de-stabilization. Moreover, close proximity between tubular

epithelium, interstitial inflammatory cells and myofibroblasts provides the ideal microenvironment for cross talk between these cell types during tissue repair.

Macrophages

Endothelial and tubular injury promotes recruitment of neutrophils, macrophages and lymphocytes in the kidney after AKI (Kinsey and Okusa, 2012; Jang and Rabb, 2014). These events amplify the inflammatory response, extending tissue injury and facilitating phagocytosis of dying cells. However, paracrine signaling from renal macrophages also plays an important role in promoting tubular repair and interstitial remodeling at later time points after AKI (Williams et al., 2010; Huen and Cantley, 2014). Signals promoting phenotypic switching from pro-inflammatory to regenerative macrophages include active phagocytosis of dying cells (including infiltrating neutrophils)(Huen and Cantley, 2014), as well as the secretion of macrophage growth factors CSF-1 and -2 by injured tubular epithelial cells (Zhang et al., 2012).

3. Using knowledge of embryonic kidney development to understand cellular repair after AKI

The coordinated mechanisms of repair involving both cell autonomous (intrinsic) and paracrine interactions between epithelium, endothelium, pericytes, and macrophages after AKI is reminiscent of the cellular interactions between parallel cell types in kidney development. I therefore reasoned that our understanding of the pathways involved in kidney development could be exploited to inform our understanding of the cellular mechanisms of repair after

AKI. To do this, we took advantage of a recent study that used genetic, cell-specific labeling techniques to interrogate the genome-wide translational expression profiles in tubular epithelium, endothelium, vascular pericytes and macrophages 24 hours after IR-AKI (Liu et al., 2014). These studies used a new technology, TRAP, which allows for the affinity purification of actively translating mRNAs associated with ribosomes isolated by affinity purification. Using a series of Cre lines to activate TRAP in different cellular compartments (Six2 Cre: epithelium; FoxD1 Cre: fibroblasts; Cdh5 Cre: endothelium; Lyz2 Cre: macrophages), they isolated cell-specific mRNAs in the intact kidney immediately after lysis. This allowed them to perform simultaneous analysis of cell-specific mRNA expression profiles without the need to separate individual cellular compartments by FACS. Using the deposited gene profile datasets from these TRAP studies, I searched for expression of intrinsic and paracrine factors involved in kidney development. The developmental genes that we used to interrogate the TRAP datasets was based on information contained in a series of recent, comprehensive reviews (Li et al., 2008; Costantini and Kopan, 2010; Cain and Rosenblum, 2011; Barak et al., 2012; Chai et al., 2013; Trueb et al., 2013; Bohnenpoll and Kispert, 2014; Halt and Vainio, 2014; Herzlinger and Hurtado, 2014; Kopan et al., 2014; Nishinakamura and Sakaguchi, 2014; Yosypiv, 2014). I only included those genes and pathways that have been shown either genetically or pharmacologically to play a functional role in regulating renal development. For each of these genes we evaluated expression of intrinsic factors in the corresponding cell types in development and injury (CM/RV/PTA and UB: epithelium; EC: endothelium; Stroma: fibroblasts, pericytes and mesangial cells), as well as expression of paracrine factors by neighboring cells (including

macrophages), reasoning that different cell types may co-opt the same paracrine responses after injury. Results are shown only for those genes that showed significantly increased expression compared with sham-operated controls adjusted for multiple comparisons ($p<0.05$). We evaluated a total of 120 genes that have been shown to regulate kidney development, of which 51 were significantly regulated in one of the four cellular compartments 24 hours after IR-AKI. Additional genes not included in this analysis include the putative renal progenitor cell markers, CD24 and CD133, which are upregulated in epithelial cells 24 hours post-AKI (CD24: 1.6 fold, $p=0.0008$; CD133: 1.4 fold, $p=0.046$). These findings confirm previous studies (discussed above), and validate the approach. Results are summarized in Tables 1 and 2 with information about developmental expression, cellular targets and function of each gene in kidney development.

Table 1: Embryonic genes regulated in epithelial and fibroblast compartments post-AKI

Data obtained by interrogating cell-specific TRAP microarray data deposited on the NCBI GEO site (GEO GSE2004) based on published work from Liu et al. JCI 2014(Liu et al., 2014). Fold change has been converted from LogFC to a linear scale, and false discovery rate p values indicated after correction for multiple comparisons. Abbreviations used: IM, intermediate mesoderm; CM: cap mesenchyme; PTA, pre-tubular aggregate; RV, renal vesicle; UB, ureteric bud; S, stroma; PTC, proximal tubular epithelium; GN, glomerular; TF, transcription factor; AF, adhesion factor; ECM, extracellular matrix protein; GF, growth factor; RTP, receptor tyrosine phosphatase; HH, Hedgehog; RTK, receptor tyrosine kinase; RAS, renin angiotensin system; Ang, angiopoietin; AGM, axonal guidance molecule

Table 1.

Six2 Cre (CM-derived epithelial cells)						
Gene	**Fold-change**	**adj.P.Val**	**Expression**	**Cell targets**	**Function**	**Pathway**
C-Myc	6.47637943	0.00011547	CM/RV/Tubules	CM	CM growth	TF
Cdh6	3.30745837	0.0001129	RV	RV	RV patterning	AF
ECM-1	3.2546443	0.00019361	UB	UB	UB branching	ECM
PDGFb	2.0151227	0.00694948	EC	Pericyte	Stabilize GN caps	GF
Spry1	1.94634596	0.00015268	UB	UB	UB branching	RTP
Jag1	1.72325331	0.00421987	RV	RV	RV patterning	Notch
Notch2	1.49572507	0.01560838	RV	RV	RV patterning	Notch
Pax8	1.48733756	0.00310193	IM/CM/RTA/RV	IM+RV	IM/RV patterning	TF
Gli3	1.4821436	0.00711689	CM/RV/UB	CM+Stroma	CM/UB differentiation	HH
Notch1	1.34278799	0.01701	RV	RV	RV patterning	Notch
N-Myc	1.30284298	0.01844742	CM/RV	CM+UB	CM/UB growth	TF
Ret	1.23678787	0.02024422	UB	UB	UB branching	RTK
Fgf20	-1.1817532	0.02888695	CM	CM	CM maintenance	FGF
Agtr1b	-1.1879282	0.03005054	UB/S	UB	UB branching	RAS
Fgfrl1	-1.206714	0.03634663	RV	RV	RV patterning	FGF
Gli2	-1.2159333	0.03088384	CM/RV/UB/UB S	Stroma	UB S differentiation	HH
Fz4	-1.2808667	0.00939375	UB/RV	UB	UB branching	Wnt
Vegfa	-1.3918525	0.01269938	Podocyte	EC	Patterning GN caps	VEGF
Agt	-1.5161001	0.03699661	PTC/UB/S	UB	UB branching	RAS
Vegfb	-1.553122	0.00400948	S	UB	UB branching	VEGF
Ang1	-1.6218783	0.00362977	Podocyte	EC	Stabilize GN caps	Ang
Ptch1	-1.6721007	0.0014819	UB stroma	Stroma	UB S differentiation	HH
Brn1	-1.7184929	0.00099984	RV	RV	RV patterning	TF
Npnt	-1.7686685	0.02406516	UB	UB	UB branching	ECM
Fgf9	-1.7927826	0.00152379	UB	CM	CM maintenance	FGF
Lhx1	-1.8443451	0.00027397	IM/RV	IM+RV	IM/RV patterning	TF
Agtr1a	-2.2761045	0.00124142	UB/S	UB	UB branching	RAS
CXCL12	-2.6526394	0.00002912	S/podocyte	EC	Patterning (PT/GN caps)	Chemokine
Gfra1	-3.31917	0.00621682	UB	UB	UB branching	RTK
sfrp1	-4.9082995	0.0000218	S	UB/RV	RV diff/UB branching	Wnt
FoxD1 Cre (vascular pericyte and fibroblasts)						
Gene	**Fold-change**	**adj.P.Val**	**Expression**	**Cell targets**	**Function**	**Pathway**
Raldh2	6.23418194	0.000279	S	UB	UB branching	RA
Sox9	5.47613442	0.000783	UB	UB	UB branching	TF
fgf7	2.13673507	0.03953	CM	UB	UB branching	FGF
PDGFb	0.75471778	0.042824	EC	Pericyte	Stabilize GN caps	GF
Pbx1	-1.3676847	0.012269	CM/stroma	UB/S	UB branching/stroma	TF
fgf9	-1.4431291	0.026046	UB	CM	CM maintenance	FGF
Smo	-1.4610955	0.005545	CM/UB stroma	CM/S	CM diff/UB stroma	HH
Ptch1	-1.6245434	0.006334	UB stroma	Stroma	UB S differentiation	HH
Vegfb	-1.6400474	0.039702	S	UB	UB branching	VEGF
Ang1	-1.9196234	0.016024	Podocyte	EC	Stabilize GN caps	Ang
CXCL12	-3.9407699	0.000787	S/podocyte	EC	Patterning (PT/GN caps)	Chemokine
Sfrp1	-3.9416509	0.000879	S	UB/RV	RV diff/UB branching	Wnt

Cdh5 Cre (endothelial cells)						
Gene	**Fold-change**	**adj.P.Val**	**Expression**	**Cell targets**	**Function**	**Pathway**
CXCR4	3.38490297	0.0000307	EC	EC	Patterning (PT/GN caps)	Chemokine
Dll1	1.7276432	0.00193236	RV	RV	RV patterning	Notch
Jag1	1.62960485	0.01750718	RV	RV	RV patterning	Notch
wnt11	1.6030653	0.00784358	UB	UB	UB branching	Wnt
ECM-1	1.55669771	0.01539723	S	UB	UB branching	ECM
Vegfa	1.51089858	0.03139186	Podocyte	EC	Patterning GN capillary	VEGF
Raldh2	1.44492489	0.01764699	S	UB	UB branching	RA signaling
wnt4	1.40643807	0.03269299	PTA	PTA/RV	RV patterning	Wnt
CV-2	1.34642046	0.00553672	CM/RV	CM	CM maintenance	BMP
Bmp4	1.30770344	0.00310936	Podocyte	MC	Stabilize GN capillary	BMP
Fat4	1.25212556	0.00998643	S	CM	CM differentiation	Hippo
fgf8	1.24991241	0.04705277	RV	RV	RV patterning	FGF
fgf9	1.18869368	0.00979009	UB	CM	CM maintenance	FGF
Bmp7	1.17549189	0.04114582	CM/UB	CM	CM maintenance	BMP
wnt9b	1.15094784	0.0273861	UB	CM	CM differentiation	Wnt
Sema3a	-1.535023	0.02025937	Podocyte/UB	EC/UB	Stabilize GN cap/UB branch	AGM
Sfrp1	-1.7230822	0.00503227	S	UB/RV	RV diff/UB branching	Wnt
Vegfb	-1.9495597	0.00012436	S	UB	UB branching	VEGF
Lyz2 Cre (macrophages)						
Gene	**Fold-change**	**adj.P.Val**	**Expression**	**Cell targets**	**Function**	**Pathway**
Raldh2	3.57497878	0.00005826	S	UB/S	UB branching/Survival	RA signaling
ECM-1	3.27201955	0.00196424	S	UB	UB branching	ECM
Jag1	2.66705098	0.00069502	RV	RV	RV patterning	Notch
Sema3a	2.19950257	0.043863	Podocyte/UB	EC/UB	Stabilize GN cap/UB branch	AGM
decorin	1.60938392	0.00159439	S	CM	CM maintenance	BMP
CV-2	1.44308259	0.030709	CM	CM	CM maintenance	BMP
Shh	-1.2378484	0.04062207	UB/UB stroma	Stroma	UB stroma diff	HH
wnt11	-1.254778	0.03699451	UB	UB	UB branching	Wnt
fgf9	-1.3903233	0.02143087	UB	CM	CM maintenance	FGF
Vegfb	-1.9296478	0.00049656	S	UB	UB branching	VEGF
PDGFb	-2.0934016	0.00033829	EC	Pericyte	Stabilize GN caps	GF
CXCL12	-2.1777093	0.00069552	S/podocyte	EC	Patterning (PT/GN caps)	Chemokine
Agt	-3.4983139	0.00694161	PTC/UB/S	UB	UB branching	RAS
sfrp1	-4.6992125	0.00023495	S	UB/RV	RV diff/UB branching	Wnt

Table 2: Embryonic genes regulated in endothelial cells and renal macrophages post-AKI

Data obtained by interrogating cell-specific TRAP microarray data deposited on the NCBI GEO site (GEO GSE2004) based on published work from Liu et al. JCI 2014 (Liu et al., 2014).

Developmental pathways known to play a role in cellular repair after AKI

While the TRAP data are limited to a single time point 24 hours after injury and so may fail to capture important gene signatures that are only expressed at later time points, we were able to identify a number of developmental genes and pathways that are already known to play a role in post-AKI repair.

Wnt signaling

Wnt signaling plays an essential role in regulating cell fate specification at different stages of embryonic development (Halt and Vainio, 2014). For example, Wnt9b and Wnt11 are expressed in the UB and regulate CM differentiation and early UB branching patterns, respectively. Wnt4 is expressed in early nephron progenitors (PTA and RV), and is required for growth and specification of CM-derived epithelia. However, Wnt4 is also expressed later in embryonic development in stromal mesenchyme surrounding the UB and plays a role in regulating smooth muscle differentiation (Itaranta et al., 2006). This is a common finding that the same genes may have distinct functional roles in different cell types at different stages of embryonic development. Early nephron patterning events regulated by Wnt9b and Wnt4 are mediated by canonical, β-catenin signaling (Park et al., 2007), while Wnt11 is thought to act through non-canonical pathways to regulate UB branching (Majumdar et al., 2003). In the TRAP datasets, there was a significant increase in expression of Wnt4 and Wnt9b in endothelial cells, while Wnt11 expression is down regulated in macrophages 24 hours after IR-AKI (Table 2). There was no change in expression of these, or other Wnt ligands involved in renal development (notably Wnt7b) in either

epithelial or stromal lineages. Increased expression of Wnt4 has been described in epithelial cells early after IR-AKI (Terada et al., 2003), and in interstitial myofibroblasts at later time points (DiRocco et al., 2013). However, deletion of Wnt4 in myofibroblasts has no effect on post-injury fibrosis even though conditional activation of canonical Wnt signaling in vascular pericytes promotes spontaneous renal fibrosis (DiRocco et al., 2013). This suggests that an alternative Wnt ligand may play a role in post-injury repair by activating canonical Wnt signaling. The role of Wnt4 expression in injured renal tubular epithelial cells is unknown, and there are no other published data on the regulation of Wnt9b or Wnt11. However, Wnt7b is detectable in renal macrophages 5 days after IR-AKI and regulates epithelial repair and remodeling of the extracellular matrix after injury (Lin et al., 2010). The TRAP data suggest alternate sites of Wnt ligand expression that may play a role in regulating post-AKI repair. Interestingly, the TRAP dataset indicates that Sfrp1, a secreted Wnt antagonist expressed by stromal mesenchyme that represses UB branching and RV differentiation in cultured embryonic kidney rudiments (Yoshino et al., 2001), is markedly repressed in epithelia, fibroblasts, endothelial cells and renal macrophages 24 hours after IR-AKI (Tables), suggesting an alternate mechanism to activate Wnt signaling post-AKI.

Notch signaling

Notch signaling plays an essential role in specifying proximal segments of CM-derived renal tubular epithelium (proximal tubules and glomeruli), and in determining CD cell fates (Barak et al., 2012). The Notch ligands Jag1 and Dll1, and Notch receptor, Notch1, are expressed in the mid-region of the RV (which is

thought to give rise to proximal tubules and glomeruli), while Notch2 is more widely expressed in the RV. More recently it has been shown that Notch1 and 2 also specify mesangial cell fate in the renal stroma (Boyle et al., 2014). In addition, while there are no published data on the role of Notch signaling in specification of the renal vasculature that we are aware of, Notch1 and the Notch ligand, Dll4, play an important role in regulating VEGF-dependent embryonic vascular development (Benedito and Hellstrom, 2013). In the TRAP dataset, there is increased expression of Jag1, Notch1 and Notch2 in epithelial cells, as well as increased Jag1 expression in endothelial cells and macrophages 24 hours after IR-AKI (Tables). These findings are consistent with data showing an early increase in renal expression of Jag1, Dll1, Notch1 and Notch2 after IR-AKI (Kobayashi et al., 2008; Chen et al., 2013), and increased Notch1 activation and Jag1 protein expression in proximal tubular cells after folic acid-induced kidney injury (Bielesz et al., 2010), but also suggest that Notch ligands maybe more widely expressed in different cell types after IR-AKI. Pharmacological inhibition as well as cell-specific loss and gain of function studies indicate that prolonged Notch signaling in tubular epithelial cells increases fibrosis after folic acid-induced AKI (Bielesz et al., 2010), and increases the severity of injury, inflammation and tubular cell apoptosis after IR-AKI (Huang et al., 2011; Sorensen-Zender et al., 2014). Contrasting with these findings, inhibition of Notch signaling has also been shown to delay both functional and structural recovery from IR-AKI (Chen et al., 2013). One possibility is that persistent activation of Notch signaling in epithelial cells, which occurs after folic acid-induced injury and IR-AKI in aged mice (Bielesz et al., 2010; Sorensen-Zender et al., 2014), increases tubular injury, while more transient activation of Notch

signaling in younger mice with IR-AKI may have beneficial effects that recapitulate the developmental program in kidney development. An alternative possibility that has yet to be explored is that activation of Notch signaling may play a more dynamic role in regulating capillary vascular stability after AKI. In this regard, it is interesting to note that the TRAP dataset identified a 1.4-fold increase in expression of Dll4, which plays an important role in vascular development, in endothelial cells 24 hours after IR-AKI ($p=0.0014$, data not shown in the table), and that systemic administration of Dll4 has also been shown to accelerate functional recovery after IR-AKI (Gupta et al., 2010)

BMP signaling

BMP signaling plays an important role at different stages of kidney development (Nishinakamura and Sakaguchi, 2014). BMP7, which is expressed in the CM and UB, acts in a cooperative manner with the secreted BMP co-activator, Crossveinless-2 (CV-2), to maintain CM expansion without differentiation (Blank et al., 2009; Ikeya et al., 2010). The BMP antagonist, Gremlin, is expressed in the CM, suppresses Smad signaling in the UB, facilitating UB branching (Michos et al., 2007). BMP4 is expressed in the metanephric mesenchyme (MM) prior to UB invasion where prevents ectopic UB buds (Miyazaki et al., 2000). Later BMP4 is restricted to the UB stroma where it regulates stromal differentiation into ureteric smooth muscle cells (Wang et al., 2009). BMP4 expression in podocytes also regulates Vegfa-dependent glomerular vascularization (Ueda et al., 2008), indicating that BMPs also regulate renal vascular patterning. BMP7 expression is reduced after IR-AKI (Almanzar et al., 1998; Simon et al., 1999), and while the cellular origin of BMP7 is unclear,

systemic treatment with BMP7, or the BMP7 mimetic THR-123, reduces tubular injury after IR-AKI (Vukicevic et al., 1998; Sugimoto et al., 2012). This suggests decreased BMP7 increases tubular injury. However the situation is more complex since expression of the secreted selective BMP7 antagonist, Chordin-like 1, is also down regulated in injured proximal tubular epithelial cells after IR-AKI, and this is associated with increased BMP-dependent Smad signaling in regenerating epithelial cells (Larman et al., 2009). In the TRAP dataset, BMP4 and 7-expression were both increased in endothelial cells 24 hours after IR-AKI (Table 2), but there was no change in expression of either ligand or the BMP antagonist Gremlin in any of the other cell types. Failure to detect change in BMP7 expression in epithelia post-AKI may reflect differences in the timing of injury, but localized expression in endothelial cells suggests BMP signaling may be playing a role in regulating vascular injury and/or stability after IR-AKI.

Hedgehog signaling

Hedgehog (HH) signaling has a more limited role than Wnt, Notch and BMP signaling during early kidney development, but plays an important role in regulating smooth muscle differentiation of stromal mesenchyme surrounding the UB after the embryonic kidney has undergone patterning into definitive cortex and medulla. The Hedgehog ligands Shh and Ihh are expressed in distal CD and CM-derived epithelia, respectively, but only loss of Shh expression in the CD has an effect on renal development. The HH receptor Ptch1, which acts by repressing HH pathway activation by Smo, is restricted to the stroma surrounding medullary UB (Cain et al., 2009). However, deletion of Ptch in the CD results in ectopic activation of HH signaling and reduced UB branching.

Moreover, active repression of HH responses by expression of the downstream HH repressor, Gli3, inhibits ectopic HH signaling in nephron progenitors and UB (Cain and Rosenblum, 2011). Gli3 is expressed in UB tips, CM and RV structures, and Gli3 null mice have increased expression of HH activators, Gli1 and Gli2, increased expression of the Gli targets Pax2, Sal1, Cyclin D1 and N-Myc (Hu et al., 2006), which are expressed in the CM. In addition, *Gli3* null mice have increased proliferation in the CM indicating that HH activation promotes cell proliferation. Therefore the balance of HH activators and repressors plays an important role in regulating cell proliferation and specification in kidney development. After IR-AKI, renal expression of Shh, Ptch, Gi1, Gli2 and Gli3 are increased, reaching maximal levels 7-10 days after injury (Fabian et al., 2012; Zhou et al., 2014). Shh expression is restricted to tubular epithelium, while Gli1 expression, indicative of HH responding cells, is limited to peritubular fibroblasts after IR-AKI (Zhou et al., 2014). In addition, inhibition of HH signaling with the Smo inhibitor, Cyclopamine, decreases post-injury fibrosis and myofibroblast proliferation after severe IR-AKI (Zhou et al., 2014), suggesting that delayed activation of HH signaling after IR-AKI promotes interstitial fibrosis by increasing numbers of interstitial fibroblasts. These findings contrast with the TRAP datasets in which there is reduced expression of Smo, and of Ptch1 (which is also a marker of activated HH signaling) in renal fibroblasts, and Shh expression is decreased in macrophages but unchanged in renal epithelial cells 24 hours post-AKI (Tables), suggesting that HH signaling is decreased early after IR-AKI, and only plays an active role in regulating post-injury fibrosis later.

Signaling pathways regulating angiogenesis and vascular stability

The molecular pathways regulating vascular patterning in the developing kidney are poorly understood. What little is known largely relates to the pathways coordinating the formation of glomerular capillaries (Herzlinger and Hurtado, 2014). Primitive glomerular epithelial cells (podocytes) secrete the pro-angiogenic factor, Vegfa, which promotes endothelial recruitment and proliferation in the glomerular tuft (Eremina et al., 2003), while endothelial cells secrete Pdgfb, which promotes recruitment and proliferation of mesangial cells (Lindahl et al., 1998). Analysis of the TRAP dataset indicates that epithelial expression of Vegfa is down-regulated 24 hours after IR-AKI, while expression in other cellular compartments, notably endothelial cells, is increased (Tables 1 and 2). In contrast, Pdgfb expression by endothelial cells is markedly decreased after IR-AKI. Unexpectedly, systemic inhibition of both Vegf and Pdgf signaling using soluble VegfR2 or PdgfRb, reduce delamination and proliferation of pericytes and prevent vascular rarefaction and fibrosis after IR-AKI (Lin et al., 2011). This suggests that activation of both pathways promotes vascular instability after IR-AKI. However this contrasts with an earlier study in which treatment with the Vegfa121 isoform reduced vascular rarefaction after IR-AKI (Leonard et al., 2008), suggesting that the effects of Vegfa (and possibly Pdgfb) on vascular rarefaction may be sensitive to fine changes in pathway activation post-AKI.

Other pathways identified from the TRAP datasets that regulate the formation of glomerular capillaries include the CXCL12/CXCR4 axis, which promotes the recruitment and proliferation of mesangial cells through a Pdgfb-independent mechanism (Takabatake et al., 2009), Angiopoietin 1 (Ang1), which

is expressed by podocytes, stabilizes endothelial interactions with mesangial cells by activating Tie-2, in endothelial cells (Jeansson et al., 2011), and the anti-angiogenic axonal guidance molecule, Sema3a, which inhibits glomerular endothelial proliferation (Reidy et al., 2009). The most striking changes are in the expression of CXCL12, as this is markedly down-regulated in epithelial cells, fibroblasts and macrophages, while CXCR4 is the most highly upregulated gene in endothelial cells 24 hours after IR-AKI (Tables 1 and 2). In addition, Ang1 expression is decreased in epithelial cells and fibroblasts, while Sema3a expression is decreased in endothelial cells but markedly increased in macrophages post-AKI. These findings indicate that AKI induces complex changes in a variety of signaling pathways that are known to regulate blood vessel formation in the embryonic kidney and that these are likely to be important in determining the extent of vascular injury and repair post-AKI.

Other genes and pathways that regulate kidney development

In addition to genes that have already been shown to play a role in post-AKI repair, the TRAP datasets include a number of genes that are also regulated post-AKI and suggest additional conserved developmentally important genes and pathways that may play a role in regenerative repair after AKI.

Retinoic acid (RA) signaling

Raldh2-dependent secretion of RA by the stroma regulates Ret-dependent UB branching (Rosselot et al., 2010), and RA-dependent secretion of ECM1 by the stroma limits the extent of RA-dependent Ret expression to the UB tips (Paroly et al., 2013). Raldh2 is the most highly upregulated gene in renal

fibroblasts and macrophages, while ECM-1 is one of the most highly upregulated genes in epithelial cells and macrophages 24 hour post-AKI in the TRAP datasets (Tables). These findings are reinforced by another recent translational profiling study in which Collagen 1α1 Cre mice were used to activate TRAP in renal fibroblasts after UUO (Grgic et al., 2014). In these studies there was a 52-fold increase in Raldh2 mRNA expression 5 days after UUO. Activation of RA signaling is of particular interest as a candidate for mediating post-AKI regenerative repair of tubular epithelium since RA signaling plays an important role in amphibian limb regeneration (Maden, 1983; Maden, 2002), and is required for Zebrafish fin and heart regeneration after injury (Mathew et al., 2004; Kikuchi et al., 2011; Blum and Begemann, 2012).

Cell cycle regulators C-Myc and N-Myc

Myc proteins are a family of proto-oncogene transcription factors that drive cell cycle progression by up-regulating an array of different cell cycle regulators (Oster et al., 2002). C-Myc is expressed in the CM, RV and early tubular epithelium(Mugrauer and Ekblom, 1991). Conditional deletion of C-Myc in the CM (but not RV) inhibits CM proliferation (Couillard and Trudel, 2009). c-Myc is also the most highly upregulated epithelial gene in the TRAP dataset 24 hours after IR-AKI (Table 1). Similarly, N-Myc, which is expressed in the CM and RV only (Mugrauer and Ekblom, 1991), regulates both CM and UB proliferation(Bates et al., 2000), is also significantly upregulated in the TRAP epithelial dataset post-AKI. Since the mechanisms regulating post-AKI tubular proliferation are incompletely understood, this suggests a novel mechanism by which reactivation of Myc expression might promote tubular repair.

Miscellaneous genes

It is notable that very few of the classical CM specific genes (including Six1/2, Cited1, Meox1/2, Sal1/4, Crym, Eya1/4 and Gdnf) were detectable in injured epithelia in the TRAP datasets 24 hours after IR-AKI. In addition, while some marker of early RV epithelia including components of the Notch pathway and Pax8 (but not interestingly, Pax2), other classical RV markers including Wnt4 and Fgf8 were unchanged, while Lhx1 and Bnr1 were actually decreased after IR-AKI. These findings suggest that while injured epithelium takes on some of the markers of embryonic epithelial progenitor cells, this is not a complete reversal to the embryonic state, as is more likely to reflect the state of cellular dedifferentiation rather than actual reprogramming. It should also be noted that the functional role of any of the changes in expression of RV markers by epithelial cells after injury is unknown. Other developmentally regulated genes including Cadherin 6 (expressed in the RV) and Sox9 (expressed in stroma), which are highly upregulated in epithelial and fibroblast datasets, respectively (Table 1), regulate RV patterning (Mah et al., 2000), and UB branching (Reginensi et al., 2011), respectively. There is also a reduction in components of the renin angiotensin system (RAS), including Angiotensin receptors 1a/b (Atr1a/b), and angiotensinogen (Agt) in epithelial cells, and marked reduction in Agt in macrophages post-AKI (Tables 1 and 2). These components of the RAS pathway regulate UB branching during embryonic development (Yosypiv, 2014). The functional roles of these changes in developmental gene expression post-AKI are all unknown.

Conclusions for sections 2 and 3

Insights gained from the comparative analysis of the cellular and molecular programs involved in regulating normal kidney development with those activated or inhibited after AKI have informed our understanding of the mechanisms of injury and repair after AKI. These changes are not associated with complete reprogramming of cells to an embryonic state, but are associated with often, ectopic expression and activation of common pathways that may have both beneficial and detrimental effects of injury and repair after AKI. In addition, functional effects of changes in expression of embryonically regulated genes may be critically dependent both on the timing in relation to the injury and repair response, and with the balance of signaling events that are being activated at any one time point after injury. Further cell-specific time course analyses and well as cell specific loss and gain of function studies will be needed to elucidate the functional role of these pathways and gene expression patterns in AKI injury and repair.

4. Roles of macrophage infiltration after AKI

Over a period of days, macrophages infiltrate and proliferate, and change from initially M1 inflammatory phenotype increasing injury, to M2 reparative phenotype, promoting repair (Rogers et al., 2014). Depletion of macrophages during this regenerative phase reduces tubular repair and increases post-AKI fibrosis (Zhang et al., 2012). However, the mechanisms regulating this predictable and tightly coordinated transition of macrophage phenotypes are poorly understood. *In vitro* studies indicate that signals derived from TH1 lymphocytes, notably γIFN, promote expression of pro-inflammatory, M1

macrophage markers, while TH2-lymphocyte-derived factors, notably IL-4 and IL-13, induce expression of anti-inflammatory, M2 macrophage markers (Biswas and Mantovani, 2010). Competing signals from other lymphocyte subsets and cell types within the injured tissues, modify these responses so that the net effect is not an all or none switch from M1 to M2 macrophage phenotypes, but more of a continuum that is both temporally regulated, and organ and injury-dependent (Sica and Mantovani, 2012; Murray et al., 2014; Xue et al., 2014). CSF-1, which is secreted by injured renal tubular epithelium, promotes expansion of M2 macrophages post-AKI (Alikhan et al., 2011; Zhang et al., 2012). However, the effects of CSF-1 on macrophage polarization are likely to result from the expansion of committed anti-inflammatory macrophages, since there is no evidence that CSF-1 promotes M2 switching in either naïve or pro-inflammatory M1 spectrum macrophages. Therefore additional signal (s) must be required to initiate this transition from M1 to M2 macrophage phenotypes after injury. Cell culture studies suggest that this may be a factor derived from injured tubular epithelium, since cultured renal tubular epithelial cells secrete a factor that induces M2 polarization of naïve bone marrow-derived macrophages (Lee et al., 2011). The injury-dependent mechanisms regulating this renal tubular cell response *in vivo* are unknown.

5. Retinoic acid (RA) signaling in kidney development and disease

Retinoic acid (RA) signaling is an evolutionary conserved pathway that regulates diverse cellular responses critical for axial patterning and organogenesis in most metazoans (Albalat, 2009; Gutierrez-Mazariegos et al.,

2014). RA is a lipid soluble, membrane permeable, biologically active metabolite of vitamin A (retinol), which acts in an autocrine and/or paracrine fashion by activating RA receptor (RAR)-dependent transcriptional responses in the nucleus (Lefebvre et al., 2005; Theodosiou et al., 2010). The extent and duration of RA signaling is tightly controlled by the rate of RA synthesis, through the regulated and localized expression of rate limiting RA synthesizing enzymes, retinaldehyde dehydrogenases, Raldh1-4 (Marchitti et al., 2008; Kumar et al., 2012), and through RAR-dependent regulation of the RA degrading, Cyp26 family of enzymes (Cyp26A1/B1 and C1) (Ross and Zolfaghari, 2011). In mammals, RA signaling plays an essential role in regulating organ development (Rhinn and Dolle, 2012), including growth and patterning of the kidney at different stages of embryonic development (Batourina et al., 2001; Rosselot et al., 2010). Stromal and collecting duct expression of RA synthesizing enzymes Raldh2 and Raldh3, as well as widespread activation of RA signaling in the embryonic kidney (Rosselot et al., 2010), are largely repressed in the adult (Wong et al., 2012). However, reactivation of this embryonic signaling pathway in adults has long been known to playing an important role in amphibian limb regeneration(Maden, 1983; Maden, 2002), and is essential for fin and heart regeneration in zebrafish following injury (Mathew et al., 2004; Kikuchi et al., 2011; Blum and Begemann, 2012). In addition, RA signaling is reactivated following cardiac injury in humans and in mice (Bilbija et al., 2012; Bilbija et al., 2014), and plays a role in promoting nerve and lung regeneration after injury rodents (Gudas, 2012). In the kidney it well established that RA agonists reduce injury, inflammation and fibrosis when administered therapeutically in a variety of models of renal injury, including toxin and ischemia-reperfusion-induced AKI (Wagner et al., 2000;

Lehrke et al., 2002; Schaier et al., 2003; Perez et al., 2004; Kishimoto et al., 2011; Ratnam et al., 2011; Balasubramanian et al., 2012). Despite this, remarkably little was known about whether and how this pathway is normally regulated during renal injury, and what functional role activation of this pathway in the kidney plays in regulating renal injury and repair response.

In the following three chapters, we have evaluated the regulation and function of RA signaling in mouse models of AKI. We show that RA signaling is activated in injured tubular epithelial cells and in macrophages within hours of injury. We used loss and gain of function approaches to establish that activation of RA signaling reduces pro-inflammatory macrophage-dependent injury and that it enhances anti-inflammatory macrophage-dependent resolution of injury and fibrosis after AKI. In addition, we utilized an *in vivo* genetic approach to inhibit RA signaling in injured tubular epithelial cells to demonstrate that RA-dependent induction of the pro-regenerative M2 macrophages is mediated in a paracrine fashion by local secretion of RA-dependent signals from injured epithelium after AKI. These data establish for the first time that reactivation of RA signaling in the adult kidney promotes tissue repair after AKI, and demonstrate that this response is conserved in zebrafish larvae and adult mouse kidneys. These findings also establish for the first time that reactivation of RA signaling in the kidney is an injury-dependent mechanism that promotes phenotypic switching of renal macrophages post-AKI.

CHAPTER II

Methods

1. Mouse strains and genotyping

Wild type BALB/c were purchased from Charles River, *RARE-hsp68-lacZ* mice (CD-1) (Rossant et al., 1991) and *Rosa26-Stop-eYFP* (*R26R-eYFP*, C57Bl/6) (Srinivas et al., 2001), purchased from Jackson Labs. *PEPCK-Cre* mice (129svj/C57Bl/6) were kindly provided by Volker Haase (Rankin et al., 2006), and *Rosa26-LSL-RaraT403X* (*R26R-DN RAR,* 129svj/C57Bl/6) by Cathy Mendelsohn (Rosselot et al., 2010). Genotyping was performed by PCR on the ear punch biopsies using both published and unpublished allele-specific primers (Table 3).

Mouse line	Primer	Primer Sequence 5'-3'	PCR product size (bp)
PEPCK-Cre	Fw	CGGTGCTAACCAGCGTTTTC	300
PEPCK-Cre	Rv	TGGGCGGCATGGTGCAAGTT	
RARE-hsp68-lacZ	Tg- Rev	CGTGGCCTGATTCATTCC	315
RARE-hsp68-lacZ	Tg- Fwd	ATCCTCTGCATGGTCAGGTC	
R26R^{EYFP}	Fw	AAGGGAGCTGCAGTGGAGTA	
R26R^{EYFP}	Rv-Mut	CTAAAGCGCATGCTCCAGAC	469
R26R^{EYFP}	Rv-Wt	TAAGCCTGCCCAGAAGACTC	2106

Table 3. ***PCR primers used for mouse genotyping***

2. Ischemia-reperfusion-induced acute kidney injury (IR-AKI)

Surgeries were performed in male mice on a water bath-heated platform (Gaymar) at 38°C. We used 10-12 week wild type BALB/c mice, 8-10 week *PEPCK-Cre*$^{Y/+}$; *RARaDN*$^{fl/fl}$ and *PEPCK-Cre*$^{Y/+}$; *R26R-eYFP*$^{+/+}$ mice (mixed background), 16-20 week *RARE-hsp68-lacZ* mice (CD-1). Mice underwent left renal pedicle clamping (800gm pressure clamp; Roboz RS-5459) for 26 minutes for *RARE-hsp68-lacZ* mice (CD-1), 30 minutes for all other mouse strains, and tissue reperfusion confirmed before completing the surgery. Contralateral nephrectomy was performed at the same time, or after 8 days after renal pedicle clamping for long-term studies, as previously described (Skrypnyk et al., 2013). Serum creatinine was evaluated using an enzymatic cascade assay (Pointe Scientific, requires only ~7 µl of serum). Depending on the experiment, mice were treated with daily intra-peritoneal (IP) injection of 1.0 mg/kg ATRA (Sigma) or 10% DMSO/90% corn oil vehicle control starting 24 hours after injury, or 20 mg/kg BMS493 (R&D Systems) or 10% DMSO/90% corn oil vehicle starting 1 hour after renal pedicle clamping. For macrophage depletion studies, mice were treated with 40mg/kg and 20mg/kg of IP liposomal Clodrinate (LC), or liposome vehicle (LV) alone (Encapsula Nanosciences) 3 days and 1 day before injury, respectively, as described (Zhang et al., 2012). 20mg/kg BMS493 or vehicle was then administered 1 hour after clamping and kidneys harvested after 3 days. Experimental protocols were approved by Vanderbilt Institutional Animal Care and Use Committee.

3. Histologic analyses

Kidneys were harvested, 2-3mm blocks cut transversely through the cortex and medulla, and fixed in 0.2% glutaradehyde and frozen in OCT for X-Gal staining, or in 10% formalin and either frozen in OCT for X-Gal and antibody co-labeling, or mounted in paraffin for all other studies, as described (Boyle et al., 2007; Cianciolo Cosentino et al., 2013). Renal tubular injury scores (TIS) and fibrosis/collagen depositions were determined from periodic acid schiff (PAS) and Sirius red (SR) stained sections, respectively, by blinded observers. Acute and chronic TIS were evaluated directly (P.P. evaluated ATRA treatment studies, and H.Y. evaluated all other studies). To quantify Sirius red staining, we used an Olympus BX-41 microscope equipped with a polarized light filter, using Image J to quantify birefringent SR stained collagens fibril surface areas/total surface areas from digitally captured polarized light images, as described (He et al., 2010; Cianciolo Cosentino et al., 2013). β-Galactosidase (β-Gal) staining was performed on 0.2% glutaraldehyde (for hematoxylin and eosin (H&E) counterstain), or formalin (for antibody co-labeling) fixed frozen sections, as described (Boyle et al., 2007; Boyle et al., 2008). For co-labeling studies, after incubation with X-Gal substrate, sections were fixed in methanol, before antigen retrieval, blocking steps and incubation with primary and secondary antibodies and/or biotinylated lectins, as outlined below. Immunohistochemical studies were performed on formalin-fixed frozen or paraffin embedded tissue sections as previously described (Boyle et al., 2007; Boyle et al., 2008). Blocking and antibody incubation steps were performed using the universal blocking reagent (Biogenex), and auto-fluorescence reduced by incubating sections in 100mM glycine after the antigen retrieval step. Lectins, primary and secondary

antibodies, dilutions, biotin amplification, and respective antigen retrieval methods, are summarized in Tables 4 and 5. Color overlays were generated using Adobe Photoshop. For β-Gal/immunofluorescence staining overlays, the blue X-Gal color change acquired using the light microscope was pseudo-colored in white and overlaid onto simultaneously digitally acquired fluorescence images with using Image J. For quantification, 5-6 400X high power field (HPF) images were captured in the outer cortex (cortex) or outer medulla (OM), and quantified by a blinded observer (T.C., or N.S.). Tubular structures were identified from green channel auto-fluorescence signal. Results were expressed as cells/HPF, or as the ratio of stained cells, as indicated in the figure legends. Image J was used to define and quantify the average percent F4/80 positive surface areas based on data from 5-6 digitally captured HPF images.

Antigen	Source	Catalog #	Species	Dilution	Amplification	Tissue	Antigen Re	Detection
Aquaporin 1	Santa Cruz	sc-20810	Rabbit	1:100	No	paraffin	Trypsin	IF
αSMA	Millipore	CBL171	Mouse	1:100	No	paraffin	Trypsin	IF
Cleaved Caspase-3	Cell Signaling	9664	Rabbit	1:300	No	paraffin	Citrate	IF
Collagen IV	Abcam	1908	Rabbit	1:300	No	paraffin	Trypsin	IF
Dolichos Biflorus lectin	Vector Labs	B1035	-	1:200	avidin/biotin	frozen/ paraffin	Trypsin	IF
F4/80	Novus Biologicals	NB600-404	Rat	1:50	avidin/biotin	frozen	Trypsin	IF
F4/80	Novus Biologicals	NB600-404	Rat	1:1000	avidin/biotin	paraffin	Proteinase	IHC
GFP	Aves	GFP-1020	chicken	1:300	No	paraffin	Citrate	IF
GFP (zf)	Invitrogen	A11122	rabbit	1:200	No	frozen	None	IF
Ki67	Abcam	Ab15580	Rabbit	1:100	No	paraffin	Citrate	IF
Ki67	eBioscience	14-5698-82	Rat	1:50	No	paraffin	Citrate	IF
Kim1 (mouse and zf)	R&D Systems	MAB1817	Rat	1:50	No	frozen/ paraffin	Trypsin	IF
Lotus tetragonolobus lectin	Vector Labs	B1325	-	1:1000	avidin/biotin	frozen/ paraffin	Trypsin	IF
Ly-6G	BD Biosciences	551459	Rat	1:50	No	paraffin	Trypsin	IF
Myeloperoxidase	Abcam	ab16886	Mouse	1:50	No	paraffin	Trypsin	IF
PCNA	Santa Cruz	sc-25280	Mouse	1:50	No	paraffin	Citrate	IF
PCNA (zf)	Sigma	P8825	Mouse	1:3000	No	frozen	Citrate	IF
phospho-Histone H3	Cell Signaling	9701	Rabbit	1:100	No	paraffin	Citrate	IF
Raldh2	Santa Cruz	sc-22591	Goat	1:200	No	paraffin	Trypsin	IF and IHC
Raldh3	Biorbyt	orb38613	Rabbit	1:40	No	paraffin	Citrate	IF and IHC
Tamm Horsfall glycoprotein	Biomedical Technologies Inc.	BT-590	Rabbit	1:100	No	frozen/ paraffin	Trypsin	IF

Table 4. ***Primary antibodies and conditions for tissue immunostaining.***

Target species Ig	Source	Catalog #	Conjugation	Dilution
goat	Vector Labs	BA5000	biotin	1:200
mouse	Jackson ImmunoResearch	715-545-150	Dylight488	1:300
mouse (zf)	Life Technologies	A-21422	Alexa Fluor 555	1:1000
rabbit	Jackson ImmunoResearch	711-066-152	biotin	1:300
rabbit	Jackson ImmunoResearch	711-165-152	Cy3	1:600
rabbit (zf)	Life Technologies	A-11008	Alexa Fluor 488	1:1000
rat	Jackson ImmunoResearch	712-166-150	Cy3	1:300
rat	Jackson ImmunoResearch	112-095-062	FITC	1:100
rat	BD Biosciences	554014	biotin	1:200
rat (zf)	Life Technologies	A-11007	Alexa Fluor 594	1:1000
biotin	ThermoFisher Scientific	84606	NeutrAvidin Dylight550	1:300

Table 5. ***Secondary antibodies and conditions for tissue immunostaining.***

4. RNA isolation and quantitative RT-PCR

RNA was isolated from snap frozen whole kidneys and cDNA synthesis performed, as previously described (Cianciolo Cosentino et al., 2013). For renal macrophages, after perfusing the kidney with phosphate buffered saline (PBS) to remove blood, kidneys were macerated and dispersed into a single cell suspension after digestions with 2mg/ml collagenase D and 100μg/ml DNAse 1 at 37C for 1 hour, as described (Zhang et al., 2012). Macrophages were then isolated in bulk using anti-CD11b antibody-conjugated magnetic microbeads (CD11b-MACS, Miltenyi Biotec), and RNA extracted with RNA-Bee reagent (TEL-TEST). RNA quantification and integrity was determined using a NanoDrop 2000c instrument (Thermo Scientific). cDNA was amplified and labeled using SYBR Green Supermix PCR (Bio-Rad). Gene expression is expressed as relative gene expression calculated using the 2^-ddCT method, as described (Schmittgen and Livak, 2008). *Gapdh* mRNA was used as a loading control, since we see no changes in *Gapdh* mRNA expression in the kidney after injury in the IR-AKI model (Cianciolo Cosentino et al., 2013). Primer sequences along with their PrimerBank identification numbers

(Wang and Seed, 2003; Spandidos et al., 2008; Spandidos et al., 2010), or previous literature citations, are listed in Table 6.

Gene Name	Primer Sequence 5'-3'	Reference/ PrimerBank ID
Arg1	CTCCAAGCCAAAGTCCTTAGAG	Novitskaya et al., *Am J Physiol Renal Physiol*. 2014
	AGGAGCTGTCATTAGGGACATC	
a-SMA	CGCTGTCAGGAACCCTGAGA	Gorenne et al., *Circ Res*. 2006
	CGAAGCCGGCCTTACAGA	
B-actin (zf)	CGTGCTGTCTTCCCATCCA	de Groh et al., *JASN* 2010
	TCACCAACGTAGCTGTCTTTCTG	
CCL2	TTAAAAACCTGGATCGGAACCAA	Novitskaya et al., *Am J Physiol Renal Physiol*. 2014
	GCATTAGCTTCAGATTTACGGGT	
CCL3	TGCCCTTGCTGTTCTTCTCT	Novitskaya et al., *Am J Physiol Renal Physiol*. 2014
	GATGAATTGGCGTGGAATCT	
CCL4	CCATGAAGCTCTGCGTGTCTG	Oshima et al., *Gastroenterology*. 2011
	GGCTTGGAGCAAAGACTGCTG	
CCL5	CCTCACCATCATCCTCACTG	Kobayashi et al., *J Immunol*. 2012
	GAGGGAGAGGTAGGCAAAGC	
Col1a1	CCCGCCGATGTCGCTAT	Higgins et al., *J Clin Invest*. 2007
	GCTACGCTGTTCTTGCAGTGAT	
CSF1	AGTATTGCCAAGGAGGTGTCAG	Wei et al., *J Leukoc Biol*. 2009
	ATCTGGCATGAAGTCTCCATTT	
CX3CL1	ACGAAATGCGAAATCATGTGC	Novitskaya et al., *Am J Physiol Renal Physiol*. 2014
	CTGTGTCGTCTCCAGGACAA	
Cyp26b1	TGCCACCCGCGACAA	Bilbija et al., *PLoS One*. 2012
	GGAACCCTGTAGCAACCAGTGA	
GFP	GAAGTTCATCTGCACCAC	
	ATGGCGGACTTGAAGAAG	
GAPDH	TGGAGAAACCTGCCAAGTATGA	Cianciolo et al., *J Am Soc Nephrol*. 2013
	GAAGAGTGGGAGTTGCTGTTGA	
IL1-b	GCAACTGTTCCTGAACTCAACT	PrimerBank ID: 6680415a1
	ATCTTTTGGGGTCCGTCAACT	
iNOS	GTTCTCAGCCCAACAATACAAGA	Novitskaya et al., *Am J Physiol Renal Physiol*. 2014
	GTGGACGGGTCGATGTCAC	
Kim1	AAACCAGAGATTCCCACACG	Kapitsinou et al., Am J Physiol Renal Physiol. 2011
	GTCGTGGGTCTTCCTGTAGC	
Kim1 (zf)	CGCTAGAAGTAAGGCAGAA	
	CACTGTTCGTATTCGCTTTC	
Mgl1	TGAGAAAGGCTTTAAGAACTGGG	Kawao et al., *Thromb Haemost*. 2012
	GACCACCTGTAGTGATGTGGG	
MR	CAAGGAAGGTTGGCATTTGT	Novitskaya et al., *Am J Physiol Renal Physiol*. 2014
	CCTTTCAGTCCTTTGCAAGC	
Raldh1	ATACTTGTCGGATTTAGGAGGCT	PrimerBank ID: 7304881a1
	GGGCCTATCTTCCAAATGAACA	
Raldh2	TTGCAGATGCTGACTTGGAC	Kim et al., *J Biol Chem*. 2008
	TCTGAGGACCCTGCTCAGTT	
Raldh3	TCGAGAGTGGGAAGAAGGAA	Song et al., *Development*. 2009
	AGAAGACGGTGGGTTTGATG	
Raldh4	GGAGTTAGCTCAGGCAGAATCT	PrimerBank ID: 30520135a1
	AGCGAAGAACCGGAAGTTCAG	
RARb	GGGCAGATCCTGGATTTCTA	Bilbija et al., *PLoS One*. 2012
	GATGGATTGAGCAGTATGCC	
Rbp1	GCGCTCGACGTCAACGT	Bilbija et al., *PLoS One*. 2012
	GCCATCCTTGCACGATCTCTT	
Rdh10	GAACATCGTAGTGGAGTTCTTCG	PrimerBank ID: 18043920a1
	CGGTCTCCTCATTGCTCTGC	
Sdha (zf)	GAGTCTCCAATCAGTATCCAGTAGTAGA	Tang et al., *Acta Biochimica et Biophysica Sinica* 2007
	CACTGTGTGCGAGCGTGTTG	
Stra6	TGTGGTCATCGCGGACTTG	PrimerBank ID: 242332596c2
	CAACAGGCATAGGTTGCTGAA	
TGF-b1	TGGCGAGCCTTAGTTTGGA	Kapitsinou et al., Am J Physiol Renal Physiol. 2011
	TCGACATGGAGCTGGTGAAA	
Transglutaminase 2	GGTGATCCTCGCTTGAGTGT	Yamamoto et al., *J Biol Chem*. 2011
	CTCCAAATCACACCTCTCCAG	
TNF-a	CGGAGTCCGGGCAGGT	Li et al., *J Biol Chem*. 2006
	GCTGGGTAGAGAATGGATGAACA	

Table 6. ***PCR primers pairs used for qRT-PCR studies.***

5. Flow Cytometry

After perfusion of the kidneys with normal saline, the injured kidney was removed, minced into 1-2 mm fragments using fine surgical scissors, and digested in PBS containing 480 units/ml collagenase type I (Life Technologies) and 30 units/ml Dispase (BD Biosciences) for 35 min at 37°C, with intermittent agitation. After adding Fetal Bovine Serum (FBS) at a 20% final concentration to neutralize collagenase and Dispase, kidney fragments were filtered through a 40-μm mesh (Falcon; BD Biosciences). Cells were centrifuged (800 *g*, 4 minutes, 8°C) and washed once in FACS Buffer (1% FBS, 1mM EDTA in PBS) and re-suspended in FACS buffer. Cell counts were performed and 10^6 cells used for each assay. Cells were incubated for 25 minutes on ice with antibodies, then washed once and re-suspended in FACS buffer. Fluorescent conjugated antibodies and dilutions used in FACS analyses are listed in S. Table 5. Only viable cells were analyzed by gating only 7-AAD negative cells. To analyze Raldh enzymatic activity by FACS, we used ALDEFLUOR Assay kit (STEMCELL Technologies) and followed manufacturer's protocol. To analyze Arginase-1 expression by FACS, after dispersing the kidney the cell pellet was treated with an alcohol-based cell permeabilization and fixation solution according to the manufacturer's protocol. After staining, cells were analyzed using a FACS Canto II cytometer (Becton Dickinson), and offline list mode data analysis using Winlist from Verity Software House.

Antigen	fluorophore	Source	Catalog #	Species	Dilution
CD45	PE	BioLegend	103105	Mouse	0.3ul/10^6 cells
7-AAD	PerCP	BioLegend	420403	-	5.0ul/10^6 cells
F4/80	PE-Cy7	BioLegend	123113	Mouse	0.2ul/10^6 cells
Ly6C	APC	BioLegend	128015	L3 cloned CTL cells	0.3ul/10^6 cells
CD11b	Pacific Blue	BioLegend	101223	Mouse	1.0ul/10^6 cells
I-A/I-E	APC-Cy7	BioLegend	107627	Mouse	0.3ul/10^6 cells
Argenase1	FITC	R&D Systems	IC5868F	human	20.0ul/10^6 cells

Table 7. ***Antibodies and conditions for Flow Cytometry studies***

6. Statistical Analyses

Statistical analyses performed included by Student two-tailed T-Test for paired group comparisons, 1-way ANOVA for multiple between group comparisons using Tukey correction for post-hoc, pair-wise between group comparisons, and 2-way ANOVA for comparisons between treatment groups over time, using Sidak's or Bonferroni's correction for multiple between group comparisons at the same time. The minimal level of significance was set at $p \leq 0.05$ and statistical analyses performed using GraphPad Prism.

CHAPTER III

Regulation of retinoic acid signaling after acute kidney injury

This chapter was a part of publication under the tile of 'Retinoic Acid Signaling Coordinates Macrophage-Dependent Injury and Repair after Acute Kidney Injury' in *The Journal of American Society of Nephrology* in press (Chiba et al., 2015a).

1. Introduction

RA plays an essential role in kidney development (Batourina et al., 2001; Rosselot et al., 2010), and reactivation of RA signaling in adults plays an important role in limb (Maden, 1983; Maden, 2002), fin and heart regeneration in amphibians and fish (Kikuchi et al., 2011; Blum and Begemann, 2012). RA agonists reduce injury, inflammation and fibrosis in models of renal injury, including toxin and ischemia-reperfusion-AKI (IR-AKI) (Wagner et al., 2000; Lehrke et al., 2002; Schaier et al., 2003; Perez et al., 2004; Kishimoto et al., 2011; Ratnam et al., 2011; Balasubramanian et al., 2012). Despite this, the expression and its regulation of RA signaling after AKI are largely unknown.

Inflammatory M1 macrophages are recruited to the kidney where they amplify inflammatory responses and promote tissue damage. Over a period of days, these macrophages are replaced by alternatively activated M2 macrophages that promote repair (Duffield, 2010; Huen et al., 2014). The mechanisms regulating macrophage phenotypes are poorly understood. CSF-1 and CSF-2 are

secreted by tubular epithelium, and promote expansion of M2 macrophages post-AKI (Alikhan et al., 2011; Zhang et al., 2012; Huen et al., 2014). However, macrophage responses represent a spectrum of phenotypes that are likely to be dependent on multiple convergent signals after injury (Sica and Mantovani, 2012; Mantovani et al., 2013; Murray et al., 2014). Therefore, a number of additional signaling pathways are likely to converge to define macrophage phenotypes after AKI.

We now hypothesized that RA signaling is activated in macrophages after IR-AKI.

2. Results

In collaboration with our study, Dr. Neil Hukriede lab from University of Pittsburgh has shown that RA signaling increases in the kidney of zebrafish larvae after gentamicin-induced AKI model in zebrafish. Using transgenic RA reporter fish, it has been shown that RA signaling is activated rapidly in tubular epithelial cells that subsequently undergo de-differentiation and expression of the proximal tubular injury marker Kim1 after AKI.

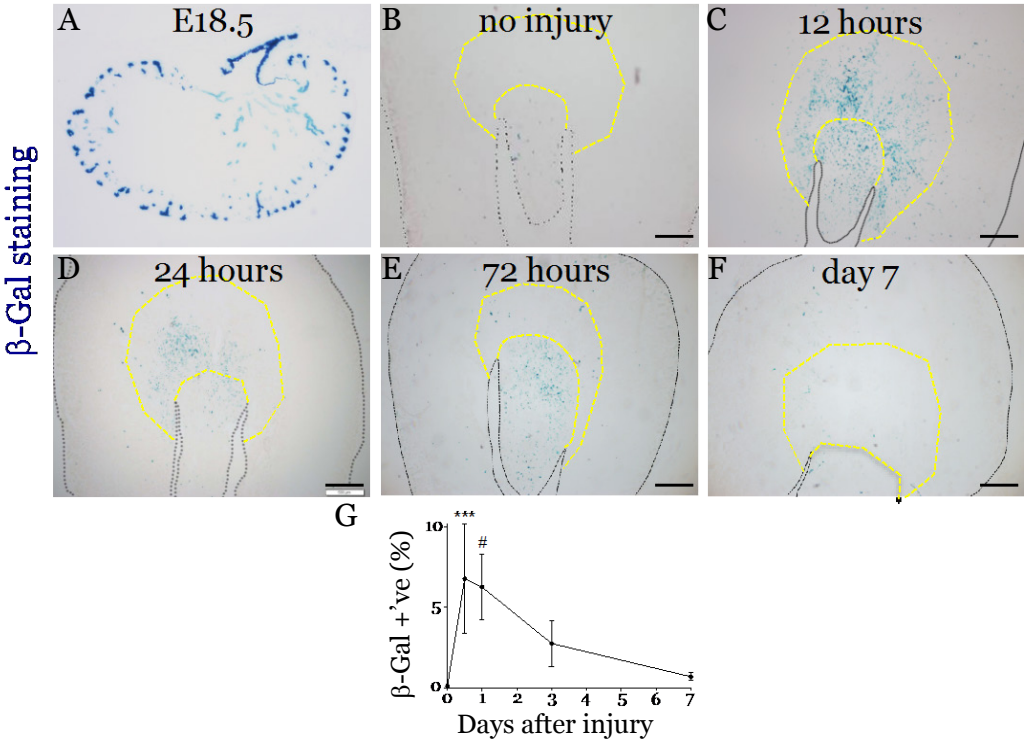
RA signaling is rapidly and transiently activated in the mouse kidney after AKI.

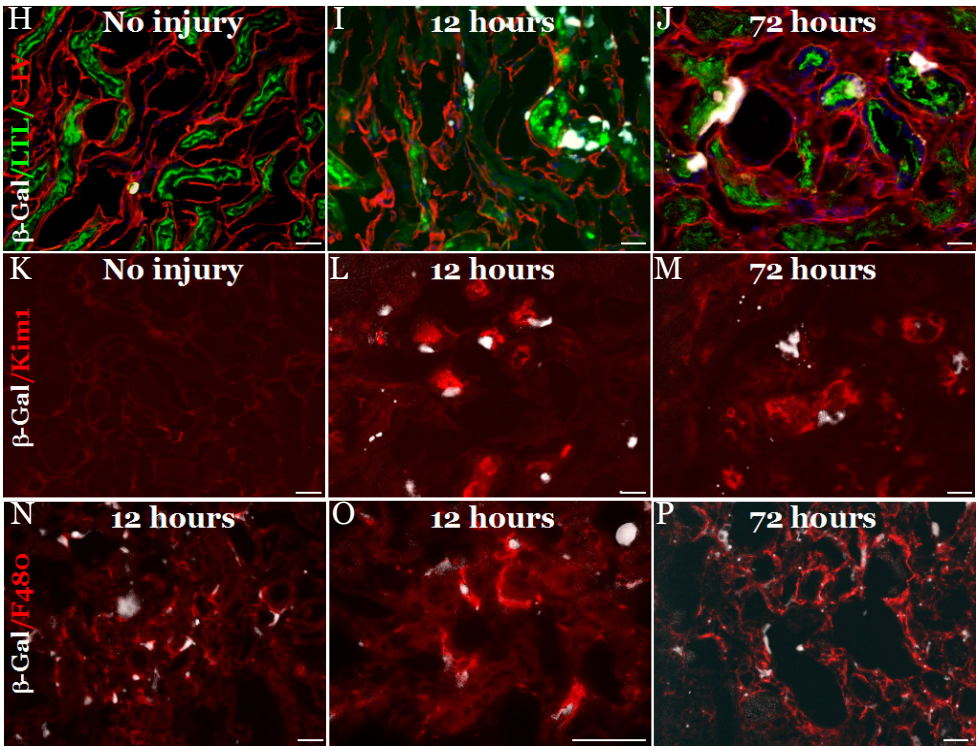
In order to determine whether RA signaling was similarly activated in the mouse kidney after AKI, we evaluated RA signaling using *RARE-hsp68-lacZ* reporter mice(Rossant et al., 1991). β-Galactosidase (β-Gal) was expressed in ureteric bud and collecting ducts (CD) in embryonic kidneys, but was restricted to occasional cells in the papilla in adults (Fig.1A/B) (Rosselot et al., 2010; Wong

et al., 2011). β-Gal was widely expressed in *RARE-hsp68-lacZ* mice, but was not detected in wild type mice 24 hours post-AKI (Fig.2). β-Gal was maximal 12-24 hours after injury, persisted at 72 hours, and returned to baseline by day 7 (Fig.1B-G). β-Gal was expressed throughout the medulla with patchy cortical expression. *RARE-hsp68-LacZ* was activated in CD epithelium (DB lectin) in uninjured and injured kidneys 72 hours post-AKI (Fig.3A/B). No PTEC β-Gal expression was detected in uninjured kidneys, but β-Gal positive LTL and Kim1 positive PTECs were detected after injury (Fig1H-M, Fig.3C/D). β-Gal was not expressed in thick limb (Tamm-Horsfall Protein, THP), or thin limb (LTL negative, Aquaporin 1, AQP1 positive) (Fig.3C/D), but was detected in macrophages post-AKI (Fig.1N-P).

Figure 1. RA signaling is activated in the kidney after IR-AKI in mice

Unilateral IR-AKI was performed in male *RARE-hsp68-lacZ* reporter mice. (A-F) RARE-dependent β-Galactosidase (β-Gal) activity in E18.5 embryonic kidney (A), in uninjured kidneys (B) and in injured kidneys at 12, 24, 72 hours and day 7 after injury (C-F), as indicated. Yellow dotted lines demarcate limits of the outer medulla. (G) Quantification of *RARE-hsp68-lacZ* reporter activity time course after injury. Percent β-Gal positive area in the outer medulla: uninjured mice (n=12), 12 hours (n=9), 24 hours (n=13), 72 hours (n=8) and day 7 (n=11) after injury. Kruskal Wallis 1-way ANOVA ($p<0.0001$) using Dunn's test for multiple comparisons with uninjured controls: ***$p<0.001$, #$p<0.0001$. (H-P) Cellular localization of β-Gal activity in *RARE-hsp68-lacZ* reporter kidneys after IR-AKI. β-Gal is pseudo-colored in white, other markers as indicated. Timing after injury, as indicated. (H-J) Proximal tubular cell marker, Lotus Tetraglonolobus Lectin (LTL, green) and Collagen IV (red). (K-M) Proximal tubular cell injury marker, Kim1 (red). (N-P) Macrophage/dendritic cell marker F4/80 (red). Black scale bars, 500μM; white bars, 50μM.


β-Gal staining
A
E18.5
B
no injury
C
12 hours
D
24 hours
E
72 hours
F
day 7
G
β-Gal +'ve (%)
Days after injury
***
#


H
No injury
I
12 hours
J
72 hours
β-Gal/LTL/CK
K
No injury
L
12 hours
M
72 hours
β-Gal/Kim1
N
12 hours
O
12 hours
P
72 hours
β-Gal/F480

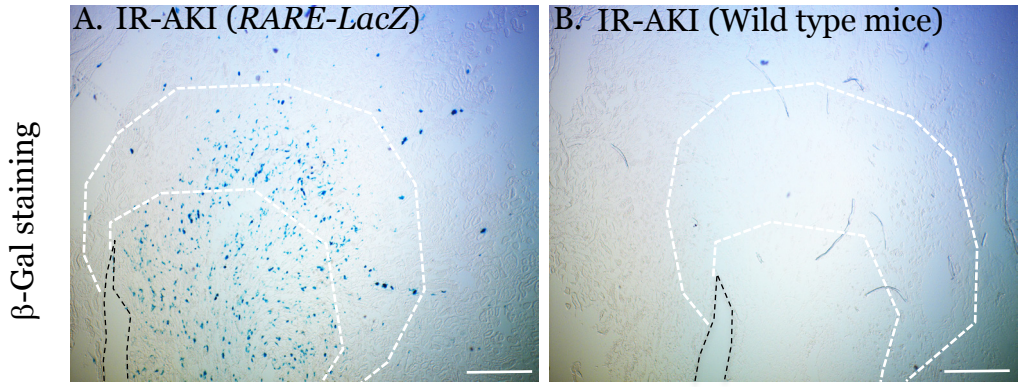


Figure 2. No β-Galactosidase activity detected in wild type mice after AKI.

Representative images showing β-Gal staining 24 hours after IR-AKI in *RARE-hsp68-LacZ* reporter mice (A) and wild type littermate (B). White dotted lines demarcate limits of the outer medulla. White scale bars, 400μM

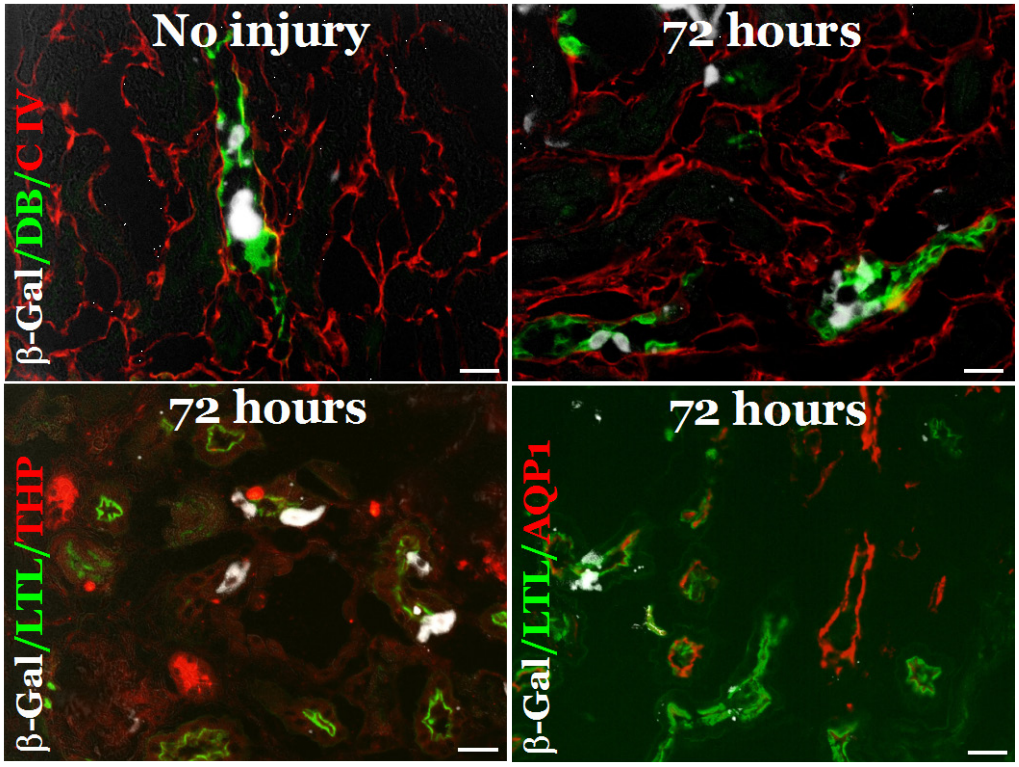


Figure 3. β -Galactosidase activity is localized to collecting ducts but not to thick limb or thin limbs after IR-AKI

β-Gal is pseudo-colored in white, other markers as indicated. (A/B) Collecting duct marker, Dolicos Biflorus Lectin (DB, green) and Collagen IV (red). (C) Loop of Henle, thick ascending limb (TAL) marker, Tamm-Horsfall protein (THP, red) with LTL (green). Yellow arrows indicate TAL segments; green arrows LTL positive PTECs. (D) Proximal tubular cell and loop of Henle, thin limb marker, Aquaporin-1 (AQP1, red) with LTL (green). Green arrows show LTL/AQP1 double positive PTECs; yellow arrows indicate LTL negative, AQP1 positive thin limb segments. White scale bars, 50μM.

Peritubular macrophages express RA synthesizing enzymes after IR-AKI

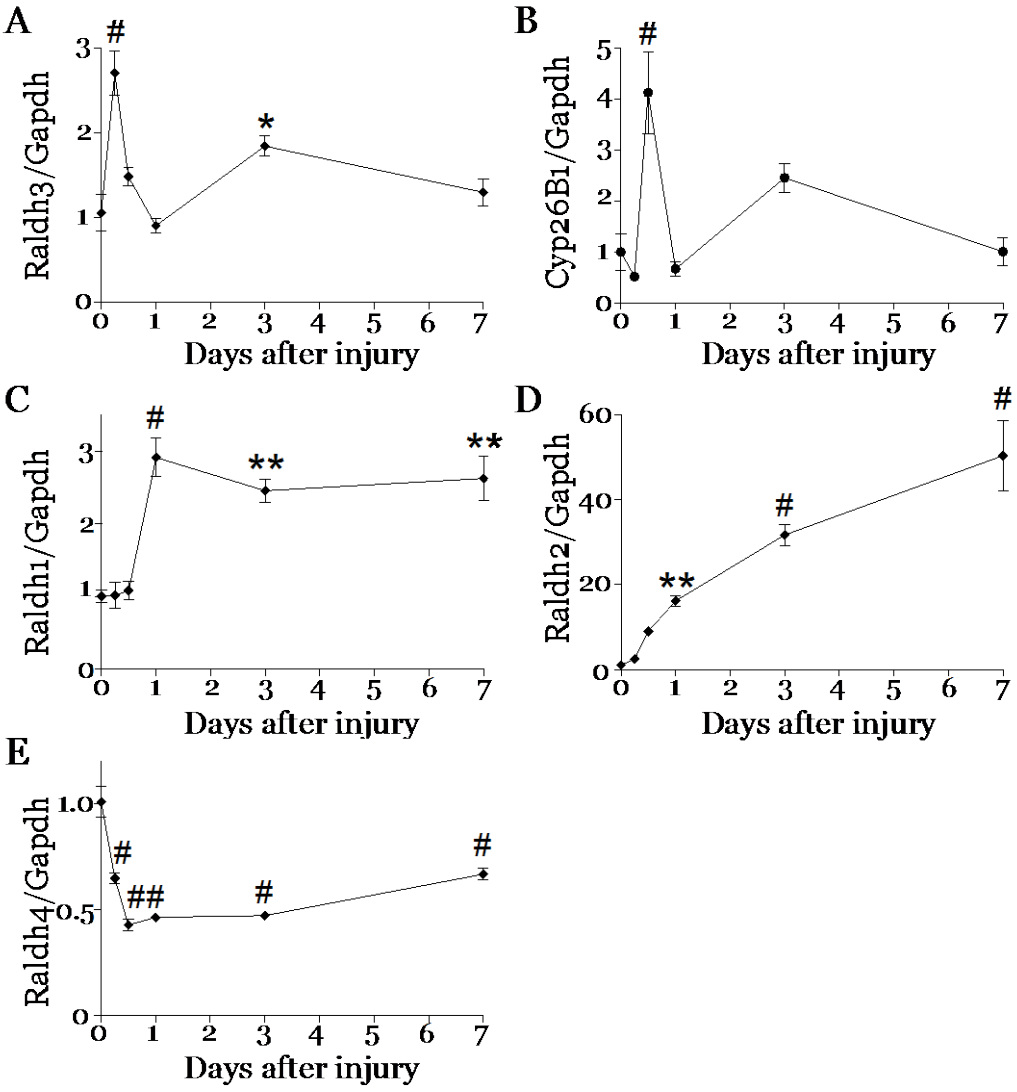
We examined expression of RA synthesizing enzymes, retinaldehyde dehydrogenases, Raldh1-4(Marchitti et al., 2008; Kumar et al., 2012). *Raldh3* mRNA paralleled the kinetics of the *RARE-hsp68-LacZ* activity, and the RAR target gene, *Cyp26B1* (Ross and Zolfaghari, 2011), after AKI (Fig.4A/B). Raldh3 was restricted to the papilla in uninjured mice (Fig.5A/B), but was expressed by peritubular cells in the outer medulla (OM) 6-24hours post-AKI (Fig.6A-E). Raldh3 was also expressed in the inner medulla with patchy expression in the cortex (Fig.5C/D). Raldh3+ cells were F4/80 negative, but 98.2 +/-0.63% (mean+/-SEM, n=4) expressed the neutrophil/macrophage marker Lys6G 12-hours after injury (Fig.6G/H). Raldh3+ cells did not express the neutrophil marker MPO (Fig.6I) (van Leeuwen et al., 2008). To establish whether Raldh3+ cells activate RA signaling, we evaluated Raldh3 and β-Gal in *RARE-hsp68-LacZ* reporter mice. Raldh3+ cells surrounded β-Gal+ tubular cells 12 hours post-AKI (Fig.6J). A number of β-Gal+ cells also co-labeled with Raldh3 (Fig.6J), suggesting paracrine Raldh3-dependent RA signaling in tubular epithelium, and autocrine RA signaling in macrophages. Since Raldh3 is an aldehyde dehydrogenase (ALDH) (Marchitti et al., 2008), we used ALDEFLUOR to identify cells with ALDH activity in the kidney by FACS (Greve et al., 2012). There were increased ALDEFLUOR high CD45/CD11b+ and CD45/Ly6C+ cells 18 hours after injury (Fig. 6K-P). 95.9% +/- 2.0% ALDEFUOR high cells were CD11b+, and 86.7% +/- 3.7% Ly6C+. To characterize CD45/Ly6C+ ALDEFLOR high cells, we first evaluated F4/80 and CD11b in CD45+ cells after injury. As previously reported (Li et al., 2008), the majority of F4/80+ cells are F4/80 high/CD11b low in uninjured kidneys (Fig. 6Q). Increased numbers of CD11b high cell express low or no

F4/80 after injury (Fig. 6R). These markers are typical of inflammatory macrophages recruited to the kidney at early time points after injury (Li et al., 2008; Li and Okusa, 2010). Using the same CD11b and F4/80 gates, 86.8%+/-0.6% ALDEFLUOR high, CD45/Ly6C+ cells are F4/80 low or negative in the injured kidney (Fig. 6S). These data are consistent with Raldh3 localization studies, suggesting the majority of Raldh3/ALDH high cells are infiltrating macrophages early after injury.

We also evaluated the expression of *Raldh1, 2* and *4* mRNAs. Of these, *Raldh2* mRNA progressively increased 1 to 7 days after injury (Fig.4C-E). Raldh2 protein was detected in peri-tubular cells at 72 hours but was undetectable 7 days after injury (Fig. 7A-D). At 72 hours Raldh2 was widely distributed throughout the kidney (Fig. 8). 87.5+/-1.5% of Raldh2 cells express F4/80 (Fig.7E), and CD11b+ renal macrophages and dendritic cells express high levels of *Raldh2* 3 days post-AKI (Fig. 7F). These data suggest that Raldh3+ macrophages recruited to injured tubules synthesize RA for the first 24 hours after AKI, and that Raldh2+ macrophages might also drive RA synthesis at later time points.

Figure 4. Retinaldehyde dehydrogenases (Raldh) 1-3 and the RA target gene, *Cy26B1* mRNAs are up-regulated in kidneys after IR-AKI

Unilateral IR-AKI was performed in male BALB/c mice and kidneys harvested at the indicated times after injury. QRT-PCR for *Raldh3* (A), *Cyp26B1* (B), *Raldh1* (C), *Raldh2* (D) and *Raldh4* (E) mRNAs relative to *Gapdh* mRNA was performed on kidney-RNA from uninjured mice (n=4), and from injured mice at 6 hours (n=7), 12 hours (n=7), 24 hours (n=10), 72 hours (n=4) and day 7 (n=5) after IR-AKI. Results expressed as mean +/- SEM fold change relative to uninjured controls. 1-way ANOVA was performed using Tukey's post-hoc correction for multiple comparisons with uninjured controls and results only indicated if 1-way ANOVA $p<0.05$: *$p<0.05$, **$p<0.01$, #$p<0.0001$.


A
Raldh3/Gapdh
Days after injury
B
Cyp26B1/Gapdh
Days after injury
C
Raldh1/Gapdh
Days after injury
D
Raldh2/Gapdh
Days after injury
E
Raldh4/Gapdh
Days after injury

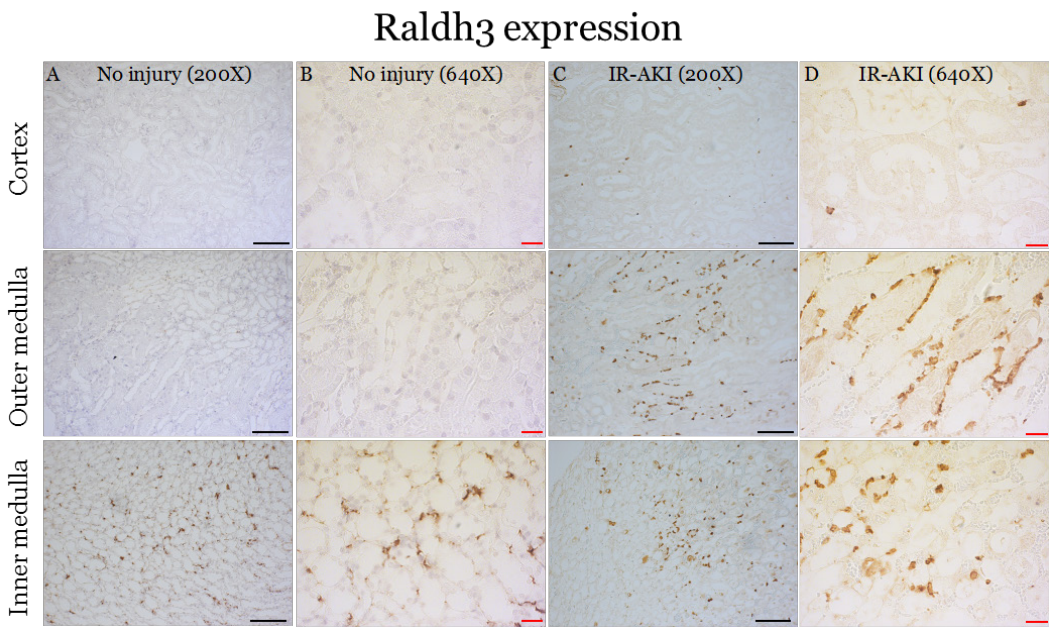


Figure 5. Raldh3 protein is localized to peritubular cells 12 hours after IR-AKI

Unilateral IR-AKI was performed in wild type BALB/c mice, and kidney sections stained with anti-Raldh3 antibody, detected using HRP/DAB substrate, counterstained with hematoxylin. (A/B) Uninjured controls. (C/D) 12 hours after IR-AKI. (A/C) 200X magnification through cortex, outer medulla and inner medulla, as indicated. (B/D) 640X magnification through cortex, outer medulla and inner medulla, as indicated. Black scale bars black 100μm, red bars 20μm.

Figure 6. Raldh3 is expressed at sites of RA signaling at early time points after IR-AKI

Unilateral IR-AKI was performed in wild type BALB/c or *RARE-hsp68-LacZ* mice, and kidneys harvested at the indicated times after injury. (A-F) Localization of Raldh3 expression in the outer medulla at 0, 6, 12, 24 and 72 hours in the outer medulla after IR-AKI. Kidney sections stained with anti-Raldh3 antibody, detected using HRP/DAB substrate, counterstained with hematoxylin. (A-E) 200X magnification. (F) 640X magnification. (G-J) Cellular localization of Raldh3 in the outer medulla after IR-AKI. Co-staining Raldh3 (red) with (G) macrophage/dendritic cells marker, F4/80 (green), (H) neutrophil and early infiltrating macrophage marker, Ly-6G (green), and (I) neutrophil marker, myeloperoxidase (MPO, green). (J) Co-localization of Raldh3 expression and RA signaling 12 hours after IR-AKI. β-Gal activity was detected in *RARE-hsp68-LacZ* reporter mice, and sections stained with Raldh3 antibodies (red). β-Gal pseudo-colored in white, green auto-fluorescence shows renal tubular structures. Green arrows indicate Raldh3 positive cells surrounding β-Gal positive renal tubular cells, yellow arrows indicate Raldh3 expressing cells that also β-Gal positive. White scale bars, 50mM, black bars, 100mM. (K-S) FACS analysis of ALDH activity in CD45+ renal leukocytes using the Aldefluor reagent. (K-M) CD11b and Aldefluor fluorescence in uninjured and injured kidney. (K/L) Representative dot plots indicating CD11b and Aldefluor high and low quadrant gates. (M) Quantification of Aldefluor high CD11b + and – cells. (N-P) Ly6C and

Aldefluor fluorescence in uninjured and injured kidney. (N/O) Representative dot plots indicating Ly6C and Aldefluor high and low quadrant gates. (P) Quantification of Aldefluor high Ly6C + and – cells. (Q/R) Representative dot plots of F4/80 and CD11b fluorescence in uninjured and injured kidneys. Gating for F4/80- (1), F4/80 low (2) and F4/80 high (3) indicated and quantified. (S) Representative dot plots of F4/80 and CD11b fluorescence in Ly6C/Aldefluor high cells (gate 2 in O) in injured kidneys. Results expressed as mean +/- SEM % of total gated cells (CD45+ or CD45/Ly6C/Aldefluor high cells, as indicated). N=3 mice per condition. (N/Q) T-test comparing CD11b+ or Ly6C+ cells from uninjured vs. injured kidneys: **$p<0.01$.

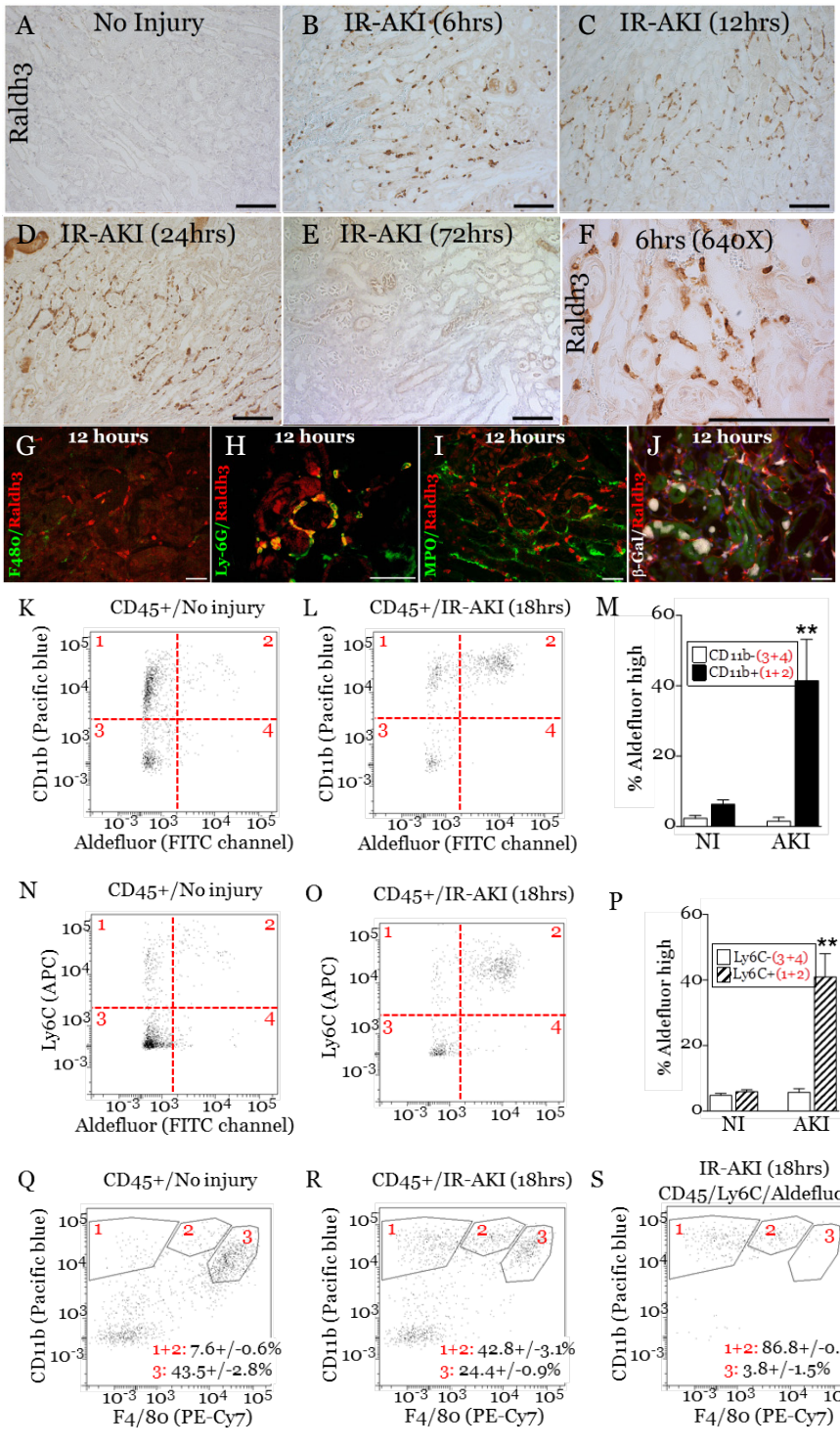

A No Injury
Raldh3
B IR-AKI (6hrs)
C IR-AKI (12hrs)
D IR-AKI (24hrs)
E IR-AKI (72hrs)
F 6hrs (640X)
Raldh3
G 12 hours
F480/Raldh3
H 12 hours
Ly-6G/Raldh3
I 12 hours
MPO/Raldh3
J 12 hours
β-Gal/Raldh3
K CD45+/No injury
L CD45+/IR-AKI (18hrs)
CD11b (Pacific blue)
Aldefluor (FITC channel)
M
% Aldefluor high
CD11b-(3+4)
CD11b+(1+2)
**
NI
AKI
N CD45+/No injury
O CD45+/IR-AKI (18hrs)
Ly6C (APC)
Aldefluor (FITC channel)
P
% Aldefluor high
Ly6C-(3+4)
Ly6C+(1+2)
**
NI
AKI
Q CD45+/No injury
R CD45+/IR-AKI (18hrs)
S IR-AKI (18hrs)
CD45/Ly6C/Aldefluor+
CD11b (Pacific blue)
F4/80 (PE-Cy7)
1+2: 7.6+/-0.6%
3: 43.5+/-2.8%
1+2: 42.8+/-3.1%
3: 24.4+/-0.9%
1+2: 86.8+/-0.6%
3: 3.8+/-1.5%

Figure 7. Raldh2 is expressed at sites of RA signaling activity 72 hours after IR-AKI

Unilateral IR-AKI was performed in wild type BALB/c mice, and kidneys harvested at the indicated times after injury. (A-D) Localization of Raldh2 expression at 0, 24, 72 hours and 7 days after IR-AKI, as indicated. Kidney sections stained with anti-Raldh2 antibody, detected using HRP/DAB substrate, counterstained with hematoxylin. (E) Co-localization of Raldh2 (red) with F4/80 positive macrophages (green) in the outer medulla 72 hours after IR-AKI. White scale bar, 50μM, black bars, 100μM. (F) Expression of *Raldh2* mRNA in renal macrophages 72 hours after IR-AKI. qRT-PCR for *Raldh2* mRNA relative to *Gapdh* control mRNA was performed on RNA extracted from renal macrophages isolated using magnetic beads coated with anti-CD11b antibodies: uninjured mice (n=3), and mice 3 days after injury (n=8). Results expressed as mean+/-SEM fold change relative to uninjured controls. 2-tailed T-Test, **p<0.01 vs. uninjured controls.

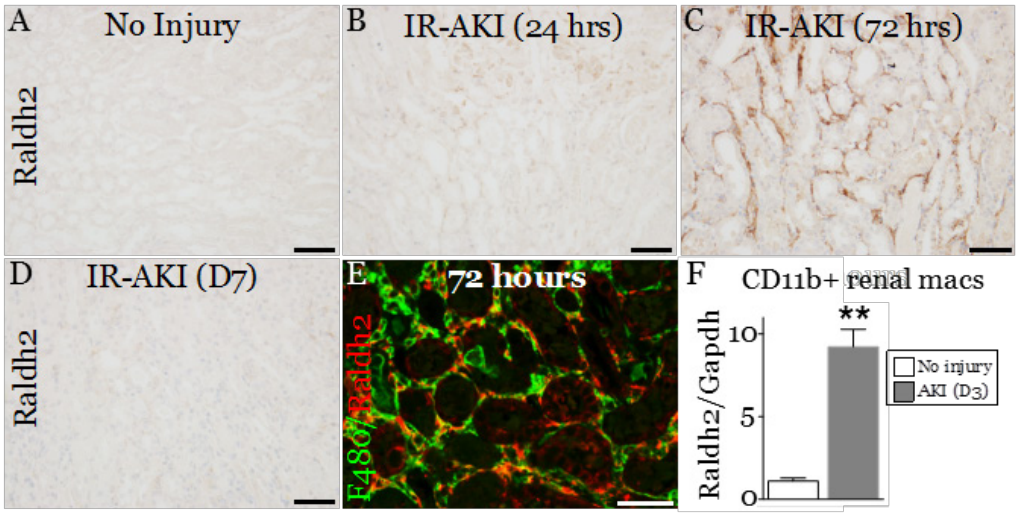
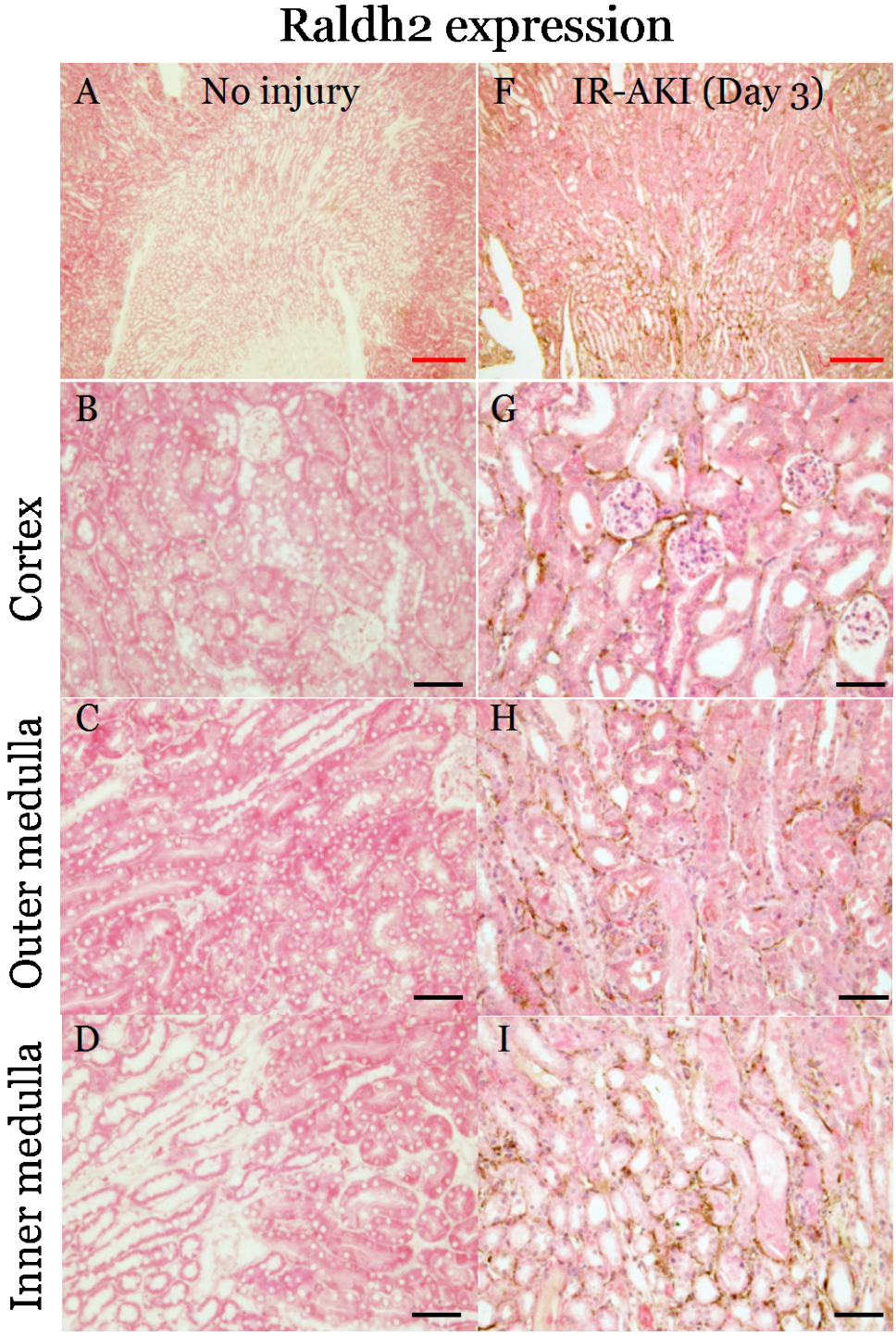

A
No Injury
Raldh2
B
IR-AKI (24 hrs)
C
IR-AKI (72 hrs)
D
IR-AKI (D7)
Raldh2
E
72 hours
F480/Raldh2
F
CD11b+ renal macs
Raldh2/Gapdh
10
5
0
**
No injury
AKI (D3)

Figure 8. Raldh2 protein is expressed in peritubular cells in injured kidney 72 hours after IR-AKI

Unilateral IR-AKI was performed in BALB/c mice, kidney sections stained with anti-Raldh2 antibody, detected using HRP/DAB substrate, counterstained with hematoxylin and eosin. (A/F) Raldh2 localization in the cortex and medulla (100X magnification) in uninjured kidneys (A) and day 3 after IR-AKI (F). (B-D, G-I) Raldh2 localization in the cortex and medulla (400X magnification), as indicated, in uninjured kidneys (B-D) and day 3 after IR-AKI (D). Red scale bars 200μm, black bars 50μm.


Raldh2 expression
A
No injury
F
IR-AKI (Day 3)
B
G
Cortex
C
H
Outer medulla
D
I
Inner medulla

3. Discussion

In these studies we show that like zebrafish larvae after gentamicin-induced AKI, RA signaling is rapidly and dynamically up-regulated in the adult mouse kidney after IR-AKI, but that unlike larval zebrafish, RA signaling is not only activated in injured PTECs, but also in renal macrophages after AKI. The origin of RA synthesis in the kidney post-AKI remains uncertain. Infiltrating macrophages express Raldh3 and have high levels of ALDH activity at early time points after injury. These cells closely associate with cells with RA signaling activity. However, Raldh2 is also expressed by macrophages, so it is possible that RA synthesis by infiltrating macrophages is replaced by mature macrophages expressing Raldh2 at later time points. Alternatively, persistent RARE-LacZ activity at later time points may be an artifact result from stabilized expression of β-Gal protein induced by early activation of the RARE, so it is possible that RA signaling is only activated early and that Raldh2 does not play a role in activating RA signaling after AKI. Furthermore, *Raldh1* mRNA increases in the kidney post-AKI, so other Raldh family members may contribute to RA synthesis. Definitive evidence as to which cells and Raldhs are synthesizing RA will require analysis of cell-specific Raldh1-3 loss of function on RARE activity.

CHAPTER IV

Functional role of retinoic acid signaling after acute kidney injury

This chapter was a part of publication under the tile of 'Retinoic Acid Signaling Coordinates Macrophage-Dependent Injury and Repair after Acute Kidney Injury' in The Journal of American Society of Nephrology in press (Chiba et al., 2015a).

1. Introduction

Essential roles of RA signaling has been demonstrated in developing kidneys (Batourina et al., 2001; Rosselot et al., 2010), and repair/regeneration models of limb (Maden, 1983; Maden, 2002), fin and heart regeneration in amphibians and fish (Kikuchi et al., 2011; Blum and Begemann, 2012). RA agonists reduce injury, inflammation and fibrosis in models of renal injury, including toxin and ischemia-reperfusion-AKI (IR-AKI) (Wagner et al., 2000; Lehrke et al., 2002; Schaier et al., 2003; Perez et al., 2004; Kishimoto et al., 2011; Ratnam et al., 2011; Balasubramanian et al., 2012). As shown in CHAPTER III, RA signaling is activated in tubular epithelial cells and macrophages within hours of injury. Furthermore, loss of function study of RA signaling in zebrafish larvae exacerbates renal tubular injury after AKI. It suggests that RA signaling is critical for recovery, liming injury and promoting PTEC proliferation in larval zebrafish

post-AKI (Chiba et al., 2015a). We now hypothesize that RA signaling plays an important role in kidney repair in mice after AKI by acting on epithelial cells and macrophages.

We show that RA reduces macrophage-dependent injury and fibrosis after AKI. Utilizing loss and gain of function as well as *in vivo* genetic approaches in mice, we demonstrate that RA regulates macrophage activation by suppressing inflammatory M1 spectrum macrophages, and indirect induction of alternatively activated M2 spectrum macrophages via RA-signaling in tubular epithelial cells.

2. Results

Inhibition of RA signaling early after IR-AKI exacerbates post-injury renal fibrosis

To evaluate the role of RA post-AKI, we used the pan-RAR inverse antagonist, BMS493 (Germain et al., 2009; Bourguet et al., 2010). BMS493 was initiated at the time of injury and continued for 72 hours (Fig. 9A). Mice were treated with 20mg/kg/day BMS493, the minimum dose inhibiting AKI-induced *RARE-hsp68-LacZ* activity (Fig. 10A/B) and up-regulation of RAR target genes, *Cyp26B1*, *RARb* and *Rbp1* (Fig. 10C-E)(Balmer and Blomhoff, 2002; Ross and Zolfaghari, 2011). BMS493 had no effect on serum creatinine (Fig.9B), but increased interstitial collagen, expression of fibrosis markers, and chronic tubular injury scores 28 days post-AKI (Fig. 9C-E). BMS493 increased renal *Kim1* mRNA, and Kim1 protein in the cortex and OM 3 days after injury (Fig. 9F-H), but had no effect on tubular apoptosis or injury by histologic scoring at this time point (Fig. 9I/J). To determine whether BMS493 has an early effect on injury, we evaluated mice undergoing unilateral IR-AKI and simultaneous contralateral nephrectomy. There was a minor increase in cortical tubular injury in BMS493

treated mice 24 hours after injury but no differences in serum creatinine (Fig. 11). Paradoxically, this minor increase in tubular injury was associated with ~30% reduction in renal macrophages in BMS493 treated mice 3 days post-AKI (Fig.9K). This was not mediated by changes in expression of macrophage growth factors or chemokines since *CSF1* mRNA was increased, and expression of chemokines implicated in macrophage recruitment after IR-AKI (Furuichi et al., 2006; Li et al., 2008), were unchanged (*CCL2* and *CCL3*) or increased (*CX_3CL1*and *CCL5*) after BMS493 treatment (Fig. 12). There was no change in tubular proliferation in BMS493 treated mice (Fig.9L-N), but was an increase in the proportion of Ki67 and phospho-histone H3 (pH3) double positive tubular cells in M-phase post-AKI (Fig.9O). This may be a consequence of the increased severity of renal injury in BMS493 treated mice (Yang et al., 2010).

Figure 9. Inhibition of RA signaling exacerbates injury and fibrosis after IR-AKI

(A) Schematic of the experiment. Studies performed in uninjured controls (n=3-6), vehicle- (n=4-8) and BMS493-treated (n=6-10) mice at day 3, and vehicle- (n=7-12) and BMS493-treated (n=7-10) mice day 28 post-AKI unless indicated in the figure. (B) Serum creatinine day 0 and 9 post-AKI. (C) Renal fibrosis day 28 after injury. Percent fibrosis in the outer medulla (OM). Images showing Sirius red staining. (D) Expression of fibrosis markers. qRT-PCR for *collagen 1α1 chain (Col1a1) and TGF-β1* mRNAs relative to *Gapdh* day 28 post-AKI. (E) Chronic tubular injury scores (OM). Injury scores at day 28. T-test: **p<0.01. (F-I) Early tubular injury after IR-AKI. (F) Tubular injury marker, *Kim1* mRNA, at day 3. (G/H) Kim1 localization 3 days after IR-AKI. (G) Representative images showing Kim1 expression. Yellow dotted lines demarcates the OM. (H) Quantification of Kim1 in the OM and cortex. T Test, *p<0.05, ***p<0.001. (I) Acute tubular injury scores. Injury scores in the OM and cortex at day 3. T-test: NS. (J) Tubular apoptosis. Cleaved Caspase-3 positive cells/high power field. 2-way ANOVA: NS treatment effect. (K) Renal macrophages. Surface area of F4/80 macrophages. 2-way ANOVA: p<0.01, vehicle vs. BMS493: #p<0.0001. (L-O) Tubular proliferation at day 3 (OM). T-test, *p<0.05. Results expressed as mean +/-SEM. (C/D/F) fold change relative to uninjured controls. 1-way ANOVA for B/C/D/F, results indicated if 1-way ANOVA: $p \leq 0.05$: *p<0.05, **p<0.01, ***p<0.001, #p<0.0001. Comparison with uninjured controls (no brackets), or vehicle and BMS493 treated mice (brackets).

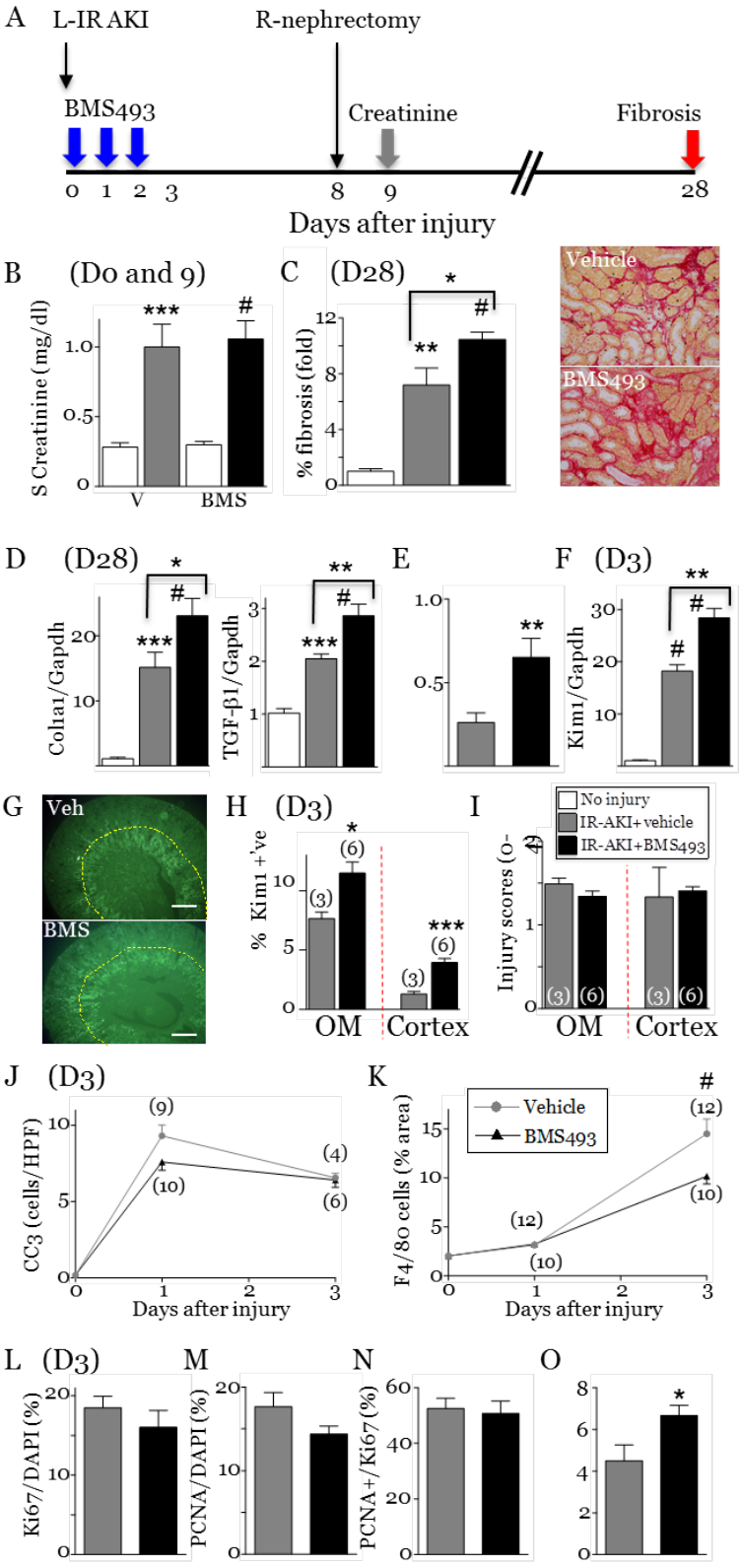
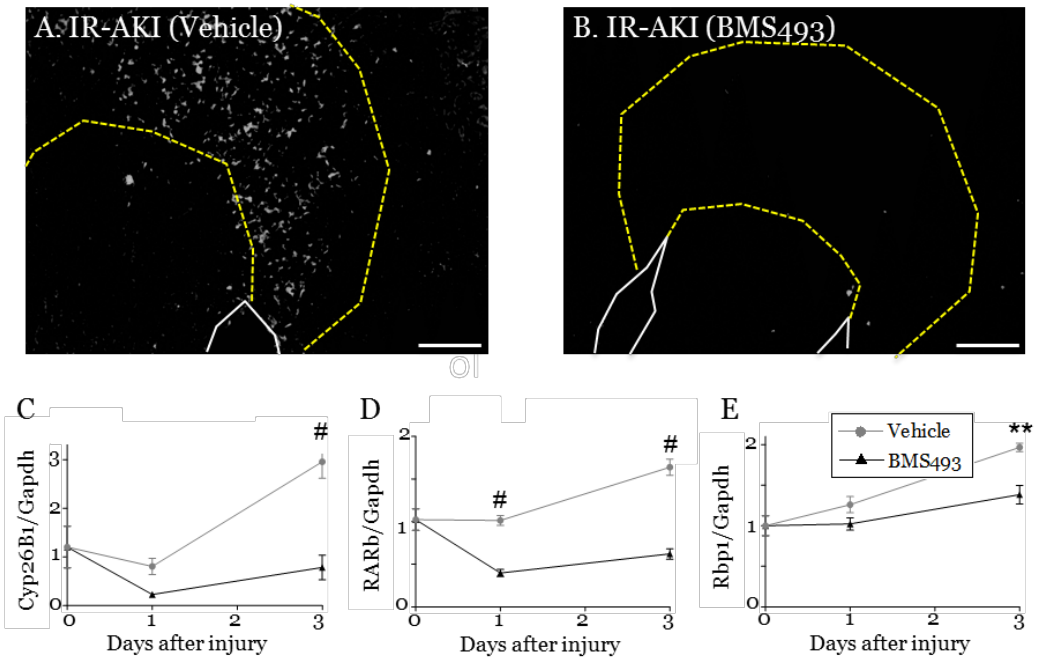

A
L-IR AKI
R-nephrectomy
BMS493
Creatinine
Fibrosis
0 1 2 3 8 9 28
Days after injury
B (Do and 9)
S Creatinine (mg/dl)
V
BMS
C (D28)
% fibrosis (fold)
Vehicle
BMS493
D (D28)
Col1a1/Gapdh
TGF-β1/Gapdh
E
F (D3)
Kim1/Gapdh
G
Veh
BMS
H (D3)
% Kim1 +ve
OM
Cortex
I
No injury
IR-AKI+ vehicle
IR-AKI+BMS493
Injury scores (0-4)
J (D3)
CC3 (cells/HPF)
Days after injury
K
Vehicle
BMS493
F4/80 cells (% area)
Days after injury
L (D3)
Ki67/DAPI (%)
M
PCNA/DAPI (%)
N
PCNA+/Ki67 (%)
O

Figure 10. BMS493 inhibits RA signaling in the kidney after IR-AKI

Unilateral IR-AKI was performed in male *RARE-hsp68-LacZ reporter* mice (A/B) or wild type BALB/c mice (C-E) treated with vehicle (10% DMSO) or 20 mg/kg BMS493 I.P. daily for 3 days starting 1 hour after injury. (A/B) β-Galactosidase activity 72 hours after unilateral IR-AKI. Untreated mice (A) and mice treated with BMS493 (B). β-Gal pseudo-colored white. Yellow dotted lines demarcate limits of the outer medulla. White scale bars, 400μM. (C-E) qRT-PCR for *Cyp26B1, Rbp1* and *Rarb* relative to *Gapdh* control mRNA on RNA extracted from uninjured (n=4), and vehicle-day 1 (n=10), day 3 (n=4), and BMS493 treated mice-day1 (n=10), day 3 (n=6) after injury. Results expressed as mean +/- SEM fold change relative to uninjured controls. 2-way ANOVA for *Cyp26B1*, $p<0.0001$; *Rbp1*, $p<0.01$; RAR β, $p<0.0001$ for vehicle vs. BMS493 effect. Sidak's correction for multiple comparison between vehicle and BMS493 treated mice at the same time points: **$p<0.01$, #$p<0.0001$.


A. IR-AKI (Vehicle)
B. IR-AKI (BMS493)
C
Cyp26B1/Gapdh
Days after injury
#
D
RARb/Gapdh
Days after injury
#
#
E
Rbp1/Gapdh
Days after injury
Vehicle
BMS493
**

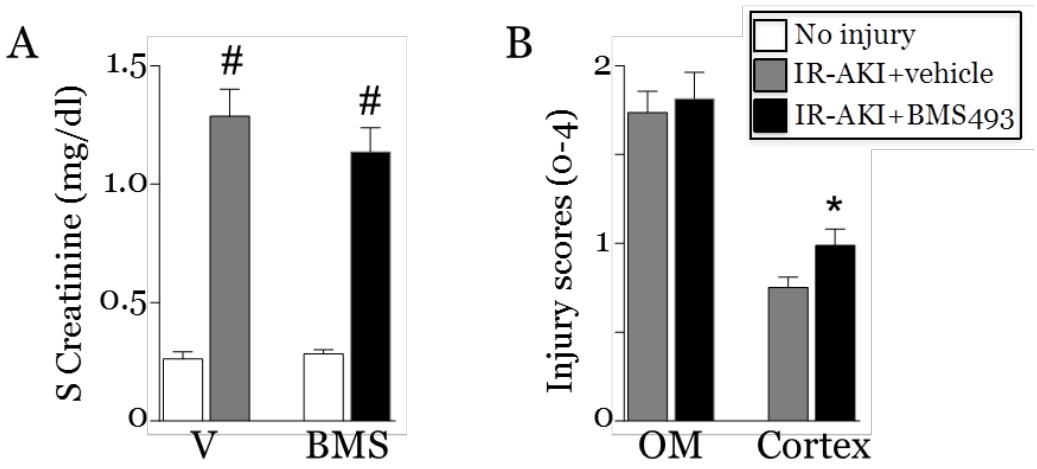
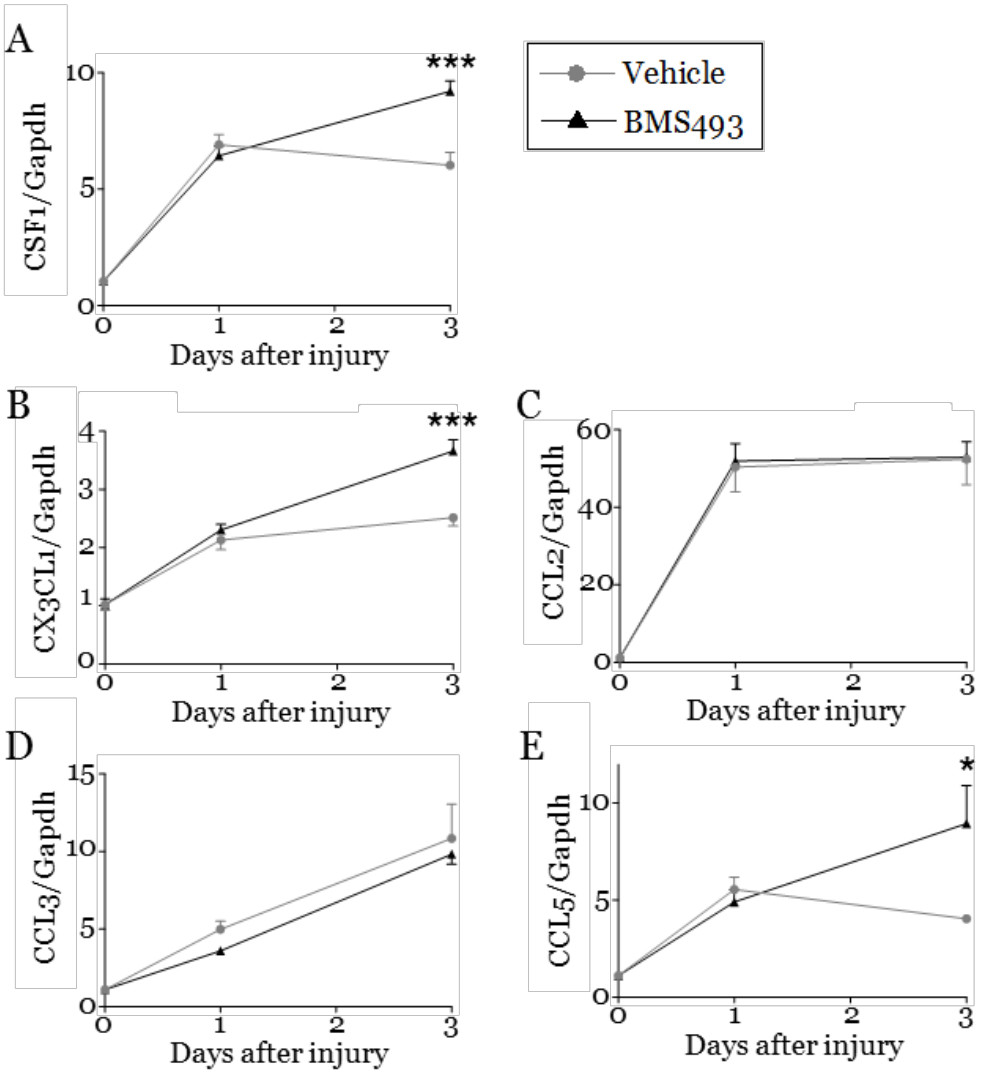


Figure 11. Early inhibition of RA signaling induces minor increase in cortical tubular injury after IR-AKI

Studies performed in uninjured controls, vehicle- and BMS493-treated mice 24 hours after unilateral IR-AKI and simultaneous contralateral nephrectomy in male BALD/c mice. Mice were treated with 20mg/kg BMS493 once at time of injury and sacrificed 24 hours later. Results expressed as means +/- SEM. 9mice per group. (A) Serum creatinine day 0 and 1 after IR-AKI. 1-way ANOVA: $p<0.0001$, post-hoc comparison with uninjured controls, #$p<0.0001$. (B) Acute tubular injury scores. Injury scores in the OM and cortex at day 1. T-test:*$p<0.05$ vs. vehicle treated injured controls.

Figure 12. BMS493 treatment increases expression of macrophage chemokines CX3CL1/Fractalkine and CCL5/Rantes, and the macrophage growth factor CSF-1/M-CSF mRNAs in the kidney after IR-AKI

qRT-PCR was performed for *CSF-1* (A), *CX_3CL1* (B), *CCL2* (C), *CCL3* (D), and *CCL5* (E) relative to *Gapdh* mRNAs on kidney-RNA. Studies were performed in uninjured (n=4), and vehicle-day 1 (n=10), day 3 (n=4), and BMS493 treated mice-day 1 (n=10), day 3 (n=6) after injury. Results expressed as mean +/- SEM fold change relative to uninjured controls. 2-way ANOVA for F4/80 cells, $p<0.01$; *CX_3CL1,* $p<0.01$; *CCL5,* $p=0.1$; *CSF-1*, $P < 0.05$ for vehicle vs. BMS493 effect. No significant between group differences for the other chemokines. Sidak's correction for comparisons between vehicle and BMS493 treated mice at the same times: *$p<0.05$, ***$p<0.001$, #$p<0.0001$.


A
CSF1/Gapdh
***
Days after injury
Vehicle
BMS493
B
CX3CL1/Gapdh
***
Days after injury
C
CCL2/Gapdh
Days after injury
D
CCL3/Gapdh
Days after injury
E
CCL5/Gapdh
*
Days after injury

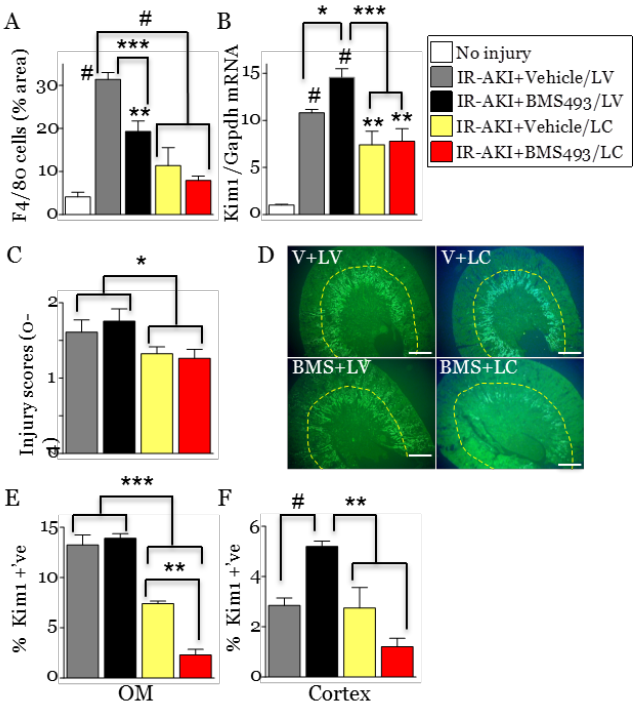
Increased injury after inhibiting RA signaling is dependent on renal macrophages post-AKI

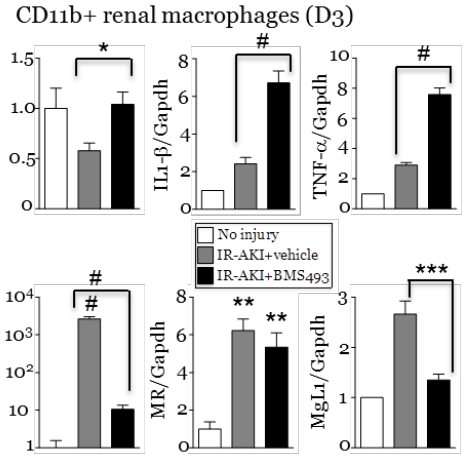
To determine whether BMS493 effects are dependent on macrophages, we used liposomal clodronate to deplete macrophages before renal injury(van Rooijen and Hendrikx, 2010). Liposomal clodronate reduced F4/80+ renal macrophages/dendritic cells by ~70% (Fig.13A), and reduced renal *Kim1* mRNA and tubular injury scores by ~25% 3 days post-AKI (Fig.13B/C). This indicates that macrophages mediate a subset of renal tubular injury responses after IR-AKI. BMS493 increased *Kim1* mRNA, and Kim1 protein was increased in the cortex 3 days post-AKI, effects that were lost after treatment with liposomal clodronate (Fig.13B-F). Thus renal macrophages are required to mediate BMS493-dependent effects on renal injury. To determine how BMS493 regulates macrophage-dependent injury, we examined renal macrophage markers. There was increased expression of M1 spectrum markers, *iNOS*, *IL1β* and *TNFα*, and decreased M2 markers *Arg1* and *Mgl1*, in renal macrophages from BMS493 treated mice (Fig.13G/H). FACS analysis demonstrated an increased proportion of Ly6C and MHC Class II antigen high renal macrophages (both markers of M1 macrophages (Sica and Mantovani, 2012; Rogers et al., 2014)) in BMS493 treated mice 3 days after IR-AKI (Fig. 13I/J). Conversely there was reduced expression of intracellular Arg1 protein in renal macrophages from BMS493 treated mice (Fig. 13K). Since M1 macrophages increase injury and M2 spectrum macrophages to promote repair (Huen et al., 2014), these data suggest RA signaling regulates post-AKI injury and repair by regulating the activation of renal macrophages.

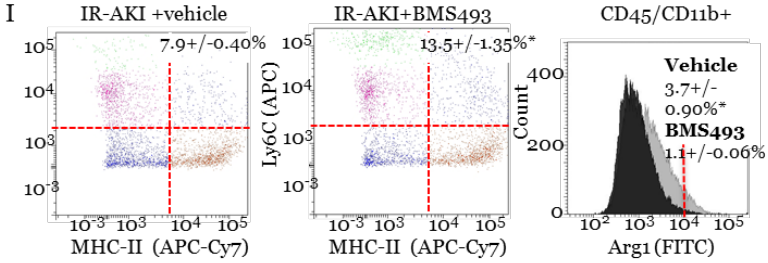
Figure 13. BMS493 increases macrophage-dependent tubular injury and deregulates macrophage polarization after IR-AKI

(A-E) Macrophage depletion prevents BMS493-dependent renal injury after IR-AKI. Unilateral IR-AKI was performed in mice pre-treated with liposomal clodronate (LC), or vehicle (LV), and randomized to receive BMS493 (BMS+LV, n=9; BMS+LC, n=6), or vehicle (V+LV, n=10; V+LC, n=4), 1 hour after injury. Kidneys harvested on day 3. (A) Liposomal clodronate depletes renal macrophages after IR-AKI. Surface area of F4/80 macrophages. (B) Tubular injury marker, *Kim1* mRNA. qRT-PCR for *Kim1*/*Gapdh* control mRNAs. (C) Acute tubular injury scores at day 3. T-test was used to compare IR-AKI with liposome vehicle and liposomal clodronate data: *p<0.05. (D-F) Kim 1 localization. (D) Representative image showing Kim1 expression. (E/F) Quantification of Kim1 in the OM and cortex. (G/H) BMS493 increases M1 and decreases M2 macrophage marker expression at day 3. qRT-PCR for M1 (G) and M2 marker (H) relative to *Gapdh* control mRNA in renal macrophages from uninjured (n=1 for *IL-1b*, *TNF-a* and *MgL1*, n=3 for *iNOS*, *Arg1* and *MR* mRNAs) and vehicle-(n=7-8) and BMS493 treated-(n=8) kidneys 3 days after injury. All results are expressed as mean +/- SEM. (B/G/H) fold change relative to controls. For analysis of I-K, *IL-1b*, *TNF-a* and *MgL1* mRNAs we used T-test to compare vehicle vs. BMS493: *p<0.05, ***p<0.001, #p<0.0001. 1-way ANOVA was used for all other studies and results only indicated if ANOVA $p \leq 0.05$: *p<0.05, **p<0.01, ***p<0.001, #p<0.0001 vs. uninjured control mice (no brackets), or vehicle vs. BMS493 mice (brackets). (I-K) FACS analysis of CD45+/CD11b+ renal

macrophages isolated 3 days after injury from vehicle or BMS493 treated mice, n=3/group. (I/J) Ly6C and MHC-II antigen expression. % Ly6C/MHC-II high indicated. (K) Intracellular Arg1 expression. % Arg1-FITC high indicated ($>10^4$ FU). T-Test comparing vehicle vs. BMS493 treatment groups (I vs. J, and K): *$p<0.05$.


A
B
F4/80 cells (% area)
Kim1 /Gapdh mRNA
No injury
IR-AKI+Vehicle/LV
IR-AKI+BMS493/LV
IR-AKI+Vehicle/LC
IR-AKI+BMS493/LC
C
Injury scores (0-4)
D
V+LV
V+LC
BMS+LV
BMS+LC
E
F
% Kim1 +'ve
OM
Cortex


CD11b+ renal macrophages (D3)
IL1-β/Gapdh
TNF-α/Gapdh
No injury
IR-AKI+vehicle
IR-AKI+BMS493
MR/Gapdh
MgL1/Gapdh


I
IR-AKI +vehicle
IR-AKI+BMS493
CD45/CD11b+
7.9+/-0.40%
13.5+/-1.35%*
Ly6C (APC)
MHC-II (APC-Cy7)
Count
Vehicle
3.7+/-
0.90%*
BMS493
1.1+/-0.06%
Arg1 (FITC)

ATRA ameliorates injury and reduces inflammatory M1 macrophage marker expression after IR-AKI

To activate RA signaling, we treated mice with all-*trans*-retinoic acid (ATRA) (Theodosiou et al., 2010). At low doses ATRA reduces fibrosis in different models of fibrosis, but at high doses may exacerbate fibrosis (Zhou et al., 2012). In uninjured mice, 10mg/kg ATRA up-regulated RAR target genes in the kidney, but 1mg/kg ATRA did not increase expression of the RAR target genes, *Rarb*, *Cy26B1* or *Stra6* mRNAs (Balmer and Blomhoff, 2002; Ross and Zolfaghari, 2011) (Fig. 14A). However, 1mg/kg ATRA increased injury-induced expression of *Rarb* and *Stra6* mRNAs 3 days after IR-AKI (Fig. 14B). 1mg/kg ATRA had no effect on RARE-LacZ reporter activation in uninjured kidneys or 24 hours after IR-AKI (Fig. 15). This indicates that low dose ATRA does not activate RA signaling in the absence of injury, and does not increase the numbers of cells with activated RA signaling after injury. At 1mg/kg/day, ATRA reduced serum creatinine (Fig.16A). By day 28 there was reduced collagen, expression of fibrosis markers and *Kim1* mRNA in the kidneys of ATRA treated mice (Fig.16B-D). *Kim1* and tubular injury scores were reduced 3 days post-AKI (Fig.16E/F). ATRA had no effect on apoptosis or renal macrophage numbers (Fig.16G/H). There were no changes in tubular proliferation (Fig.16I-K), but M-phase arrest was reduced with ATRA (Fig.16L). ATRA reduced expression of *IL1β* and *TNF-α* mRNAs (Fig.16M), but had minimal effect on renal M2 spectrum marker expression 3 days post-AKI (Fig.16N). This suggests that while ATRA represses M1 spectrum macrophages, M2 macrophages are not activated after treatment with ATRA.

Figure 14. 1mg/kg b.w dose of all-trans RA (ATRA) does not increase expression of RA target genes in uninjured kidney, but does in injured kidneys after IR-AKI

qRT-PCR was performed for *Rarb*, *Stra6* and *Cyp26B1* mRNAs relative to *Gapdh* control mRNAs. (A) Uninjured mouse kidneys harvested 12 hours after IP injection of 1mg/kg or 10mg/kg ATRA. Vehicle control (n=7-8), 1mg/kg ATRA (n=4), 10mg/kg ATRA (n=5). (B) Injured mouse kidneys 24 hours after IR-AKI from mice treated with 1mg/kg ATRA at the time of injury. Uninjured controls (n=4), vehicle and ATRA treated mouse kidneys 24 hours after IR-AKI (n=8/group). Results expressed asd mean +/- mean +/- SEM fold change relative to uninjured controls. 1-way ANOVA with Tukey's correction for multiple between group comparisons indicated where ANOVA $p<0.05$: *$p<0.05$, ***$p<0.001$. Comparisons with uninjured controls (no brackets), or vehicle and ATRA treated mice (brackets)

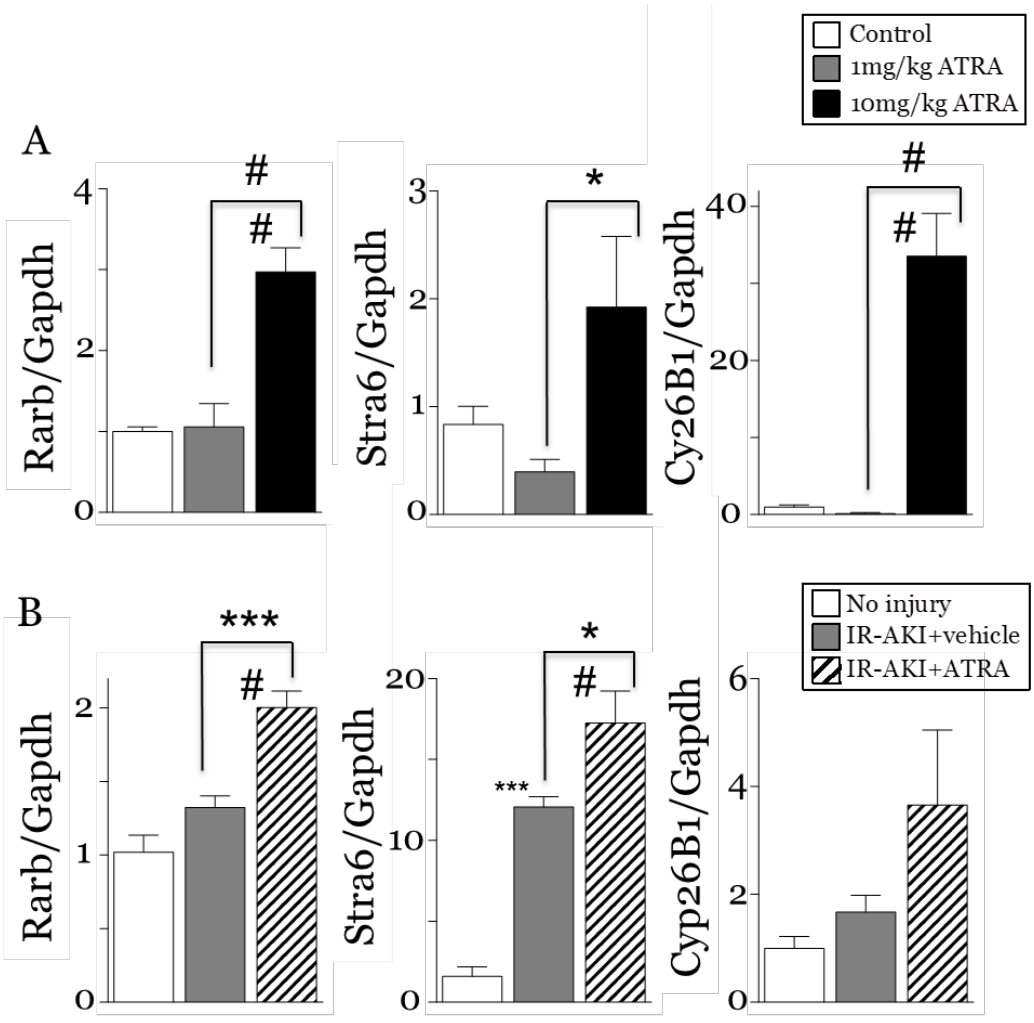

A
Control
1mg/kg ATRA
10mg/kg ATRA
Rarb/Gapdh
Stra6/Gapdh
Cyp26B1/Gapdh
B
No injury
IR-AKI+vehicle
IR-AKI+ATRA
Rarb/Gapdh
Stra6/Gapdh
Cyp26B1/Gapdh

Figure 15. ATRA treatment does not increase *RARE-hsp68-lacZ* reporter activation in the kidney

(A/B) Male *RARE-hsp68-lacZ* reporter mice were treated with vehicle (A) or 1mg/kg ATRA I.P. (B) Kidneys were harvested 12 hours after treatment for X-Gal staining. (C-F) β-Gal activity 24 hours after unilateral IR-AKI in *RARE-hsp68-lacZ* reporter mice. Unilateral IR-AKI was performed in mice treated with vehicle (C/E) or 1mg/kg ATRA (D/F) starting at the time of injury, and kidneys harvested 24 hours after IR-AKI for β-Gal staining. (C/D) β-Gal staining (blue). (E/F) Co-labelling β-Gal staining with F4/80. β-Gal pseudo-colored white, F4/80 staining red. White dotted lines demarcate limits of the outer medulla. Black dotted lines renal papilla. Red scale bars, 500μM, yellow scale bars, 200μM.

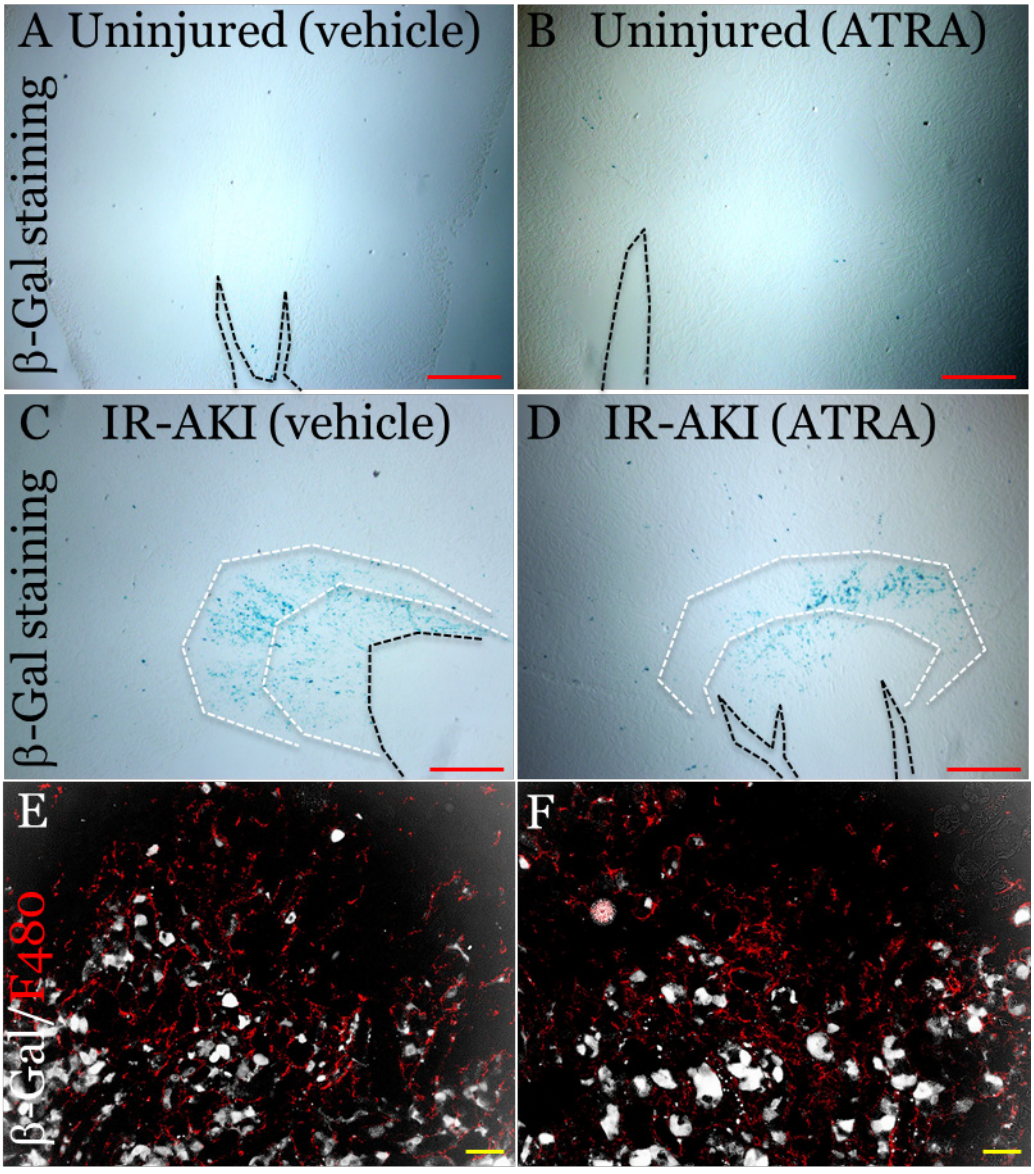
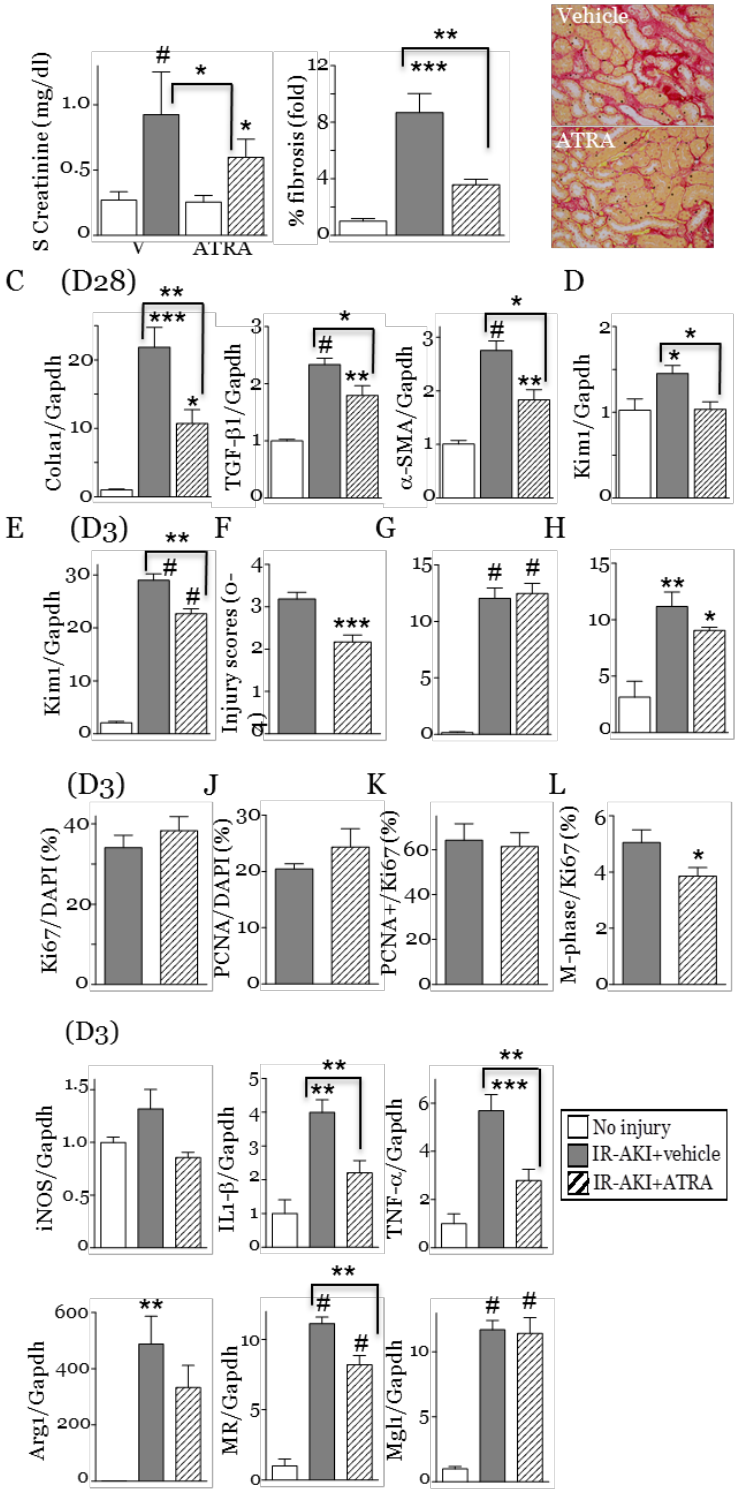

A Uninjured (vehicle)
B Uninjured (ATRA)
β-Gal staining
C IR-AKI (vehicle)
D IR-AKI (ATRA)
β-Gal staining
E
F
β-Gal/F480

Figure 16. All-trans retinoic acid (ATRA) attenuates injury and fibrosis after IR-AKI

Unilateral IR-AKI performed on male BALB/c mice with contralateral nephrectomy at day 8. Daily treatment with vehicle or ATRA started 24 hours post-injury for 7 days. Kidneys harvested at day 3 and 28. Uninjured controls (n=3-4), vehicle- (n=7-9) and ATRA-treated (n=7-8) at day 3, and vehicle- (n=6-7) and ATRA-treated (n=7) kidneys day 28. (A) Serum Creatinine 0 and 9 days post-AKI. (B/C) Renal fibrosis at day 28. (B) Percent fibrosis in the OM. Images showing Sirius red staining. (C) Expression of fibrosis markers. qRT-PCR for *Col1a1, TGF-β1* and *α–smooth muscle actin (α-SMA)* relative to *Gapdh* mRNA at day 28. (D) Tubular injury marker, *Kim1* mRNA 28 days post-AKI. qRT-PCR for *Kim1*/*Gapdh* control mRNAs. (E-G) Early tubular injury after IR-AKI. (E) *Kim1* mRNA at day 3. (F) Tubular injury scores (OM) at day 3. (G) Tubular apoptosis. Cleaved Caspase-3 positive cells/high power field at day 3. (H) Renal macrophages. Surface area of F4/80 macrophages. (I-L) Tubular proliferation at day 3 (OM). T-Test, *p<0.05. (M/N) ATRA decreases M1 macrophage marker expression at day 3. qRT-PCR for M1 (M) and M2 markers (N) relative to *Gapdh* control mRNAs. Results expressed as mean +/- SEM. qRT-PCR and fibrosis data (B) expressed as fold change relative to controls. Unless otherwise indicated, 1-way ANOVA performed. Results only indicated if ANOVA p<0.05: *p<0.05, **p<0.01, ***p<0.001, #p<0.0001. Comparison with uninjured controls (no brackets), or vehicle and ATRA treated mice (brackets).


S Creatinine (mg/dl)
% fibrosis (fold)
V
ATRA
Vehicle
ATRA
C
(D28)
Col1a1/Gapdh
TGF-β1/Gapdh
α-SMA/Gapdh
D
Kim1/Gapdh
E
(D3)
Kim1/Gapdh
F
Injury scores (0-4)
G
H
(D3)
J
Ki67/DAPI (%)
PCNA/DAPI (%)
K
PCNA+/Ki67 (%)
L
M-phase/Ki67 (%)
(D3)
iNOS/Gapdh
IL1-β/Gapdh
TNF-α/Gapdh
No injury
IR-AKI+vehicle
IR-AKI+ATRA
Arg1/Gapdh
MR/Gapdh
Mgl1/Gapdh

Inhibition of RA signaling in PTECs inhibits expression of M2 spectrum renal macrophage markers after IR-AKI

To determine whether RA signaling in PTECs mediates RA-dependent modification of M2 spectrum macrophages, we crossed *Rosa26-LSL-RaraT403X (R26R-DNRAR)* mice, which express a Cre-activated, dominant negative RAR (Damm et al., 1993; Rosselot et al., 2010), with *PEPCK-Cre* mice (Rankin et al., 2006), to generate PTEC-DNRAR mice. *PEPCK-Cre* induces efficient recombination in cortical and medullary PTECs (Fig.17) (Rankin et al., 2006). We used mice homozygous for *R26R-DNRAR* for efficient RAR inhibition, as described (Rosselot et al., 2010). PTEC-DNRAR mice have normal kidneys (Fig.18), but increased renal *Kim1* mRNA as well as Kim1 staining in the cortex compared with Cre- controls 3 days post-AKI (Fig.19A-C). There was no change in proliferation but there were increased tubular cells arrested in M phase in PTEC-DNRAR mice post-AKI (Fig.19D). Unlike BMS493 treated mice, there was reduced expression of M1 spectrum marker mRNAs for *IL1β* and *TNFα* but no change in *iNOS* mRNA expression in CD11+ cells from *R26R-DNRAR* mice post-AKI (Fig.20A). As *iNOS* is repressed in renal macrophages 3 days after injury (Fig. 13G/20A), it may not be a good marker of inflammatory macrophages in this model. There was increased expression of *Raldh2* and *3* and the RAR target genes *Rarb* and *Rbp1* in PTEC-DNRAR kidneys post-AKI (Fig.20D/E), and *Raldh3*, *Rarb, Rbp1*, and the developmentally regulated RA target *Ret* mRNAs (Balmer and Blomhoff, 2002), were also increased in renal macrophages from PTEC-DNRAR mice 3 days post-AKI (Fig. 20F). This suggests there may be a compensatory increase in RA synthesis in PTEC-DNRAR kidneys associated with increased RA signaling in renal macrophages. Since RA treatment suppresses inflammatory macrophages, this compensatory increase in RA synthesis may account for suppression of inflammatory macrophages in PTEC-DNRAR mice post-AKI. However, in

addition to effects on inflammatory macrophages, there was also a marked reduction in expression of M2 spectrum markers *Arg1*, *MR*, and *Mgl1* mRNAs 3 days post-AKI (Fig.20B). This was not associated with reduced macrophage numbers (Fig.20C). These data indicate that activation of RA signaling in PTECs promotes alternative activation of macrophages after AKI.

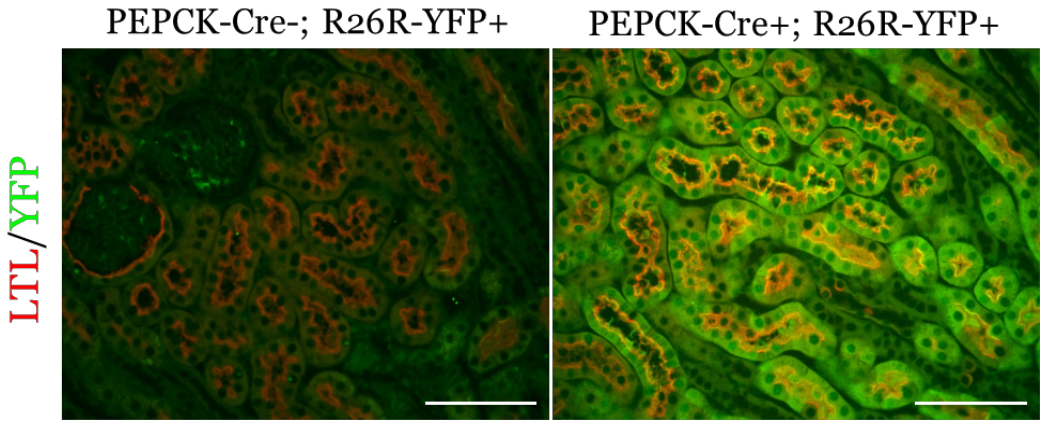


Figure 17. PEPCK-Cre shows efficient Cre dependent recombination in both cortical and medullary PTECs

Male *PEPCK-Cre*$^{/-}$ and *PEPCK-Cre*$^{/+}$ mice were crossed with *Rosa26R-YFP* (*R26R-YFP*) reporter mice to generate homozygous *PEPCK-Cre*$^{/-}$ *and PEPCK-Cre*$^{/+;}$ *R26R-YFP*$^{+/+}$ mice. Kidneys were harvested at 8 weeks of age and YFP visualized directly (green), counterstained red with LTL lectin as a marker of PTECs. Scale bars 100μm

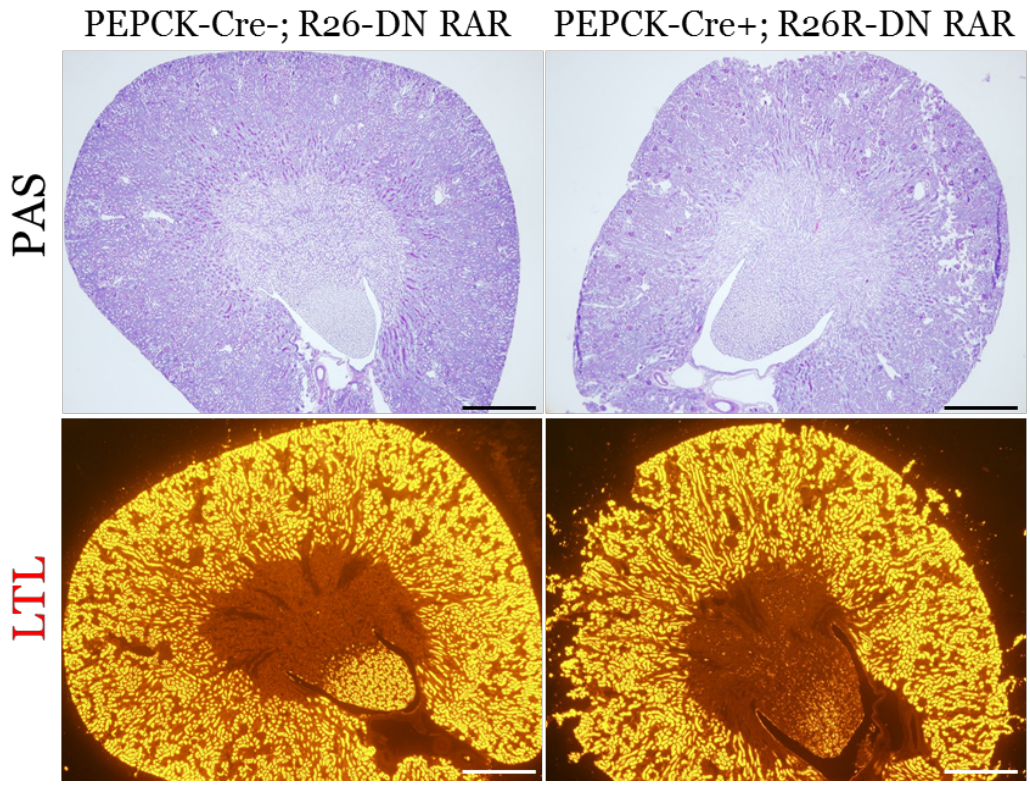


Figure 18. PTEC DN RAR mice have normal kidneys

Kidneys from Cre- and Cre+ PTEC DN RAR mice were harvested at 8 weeks of age and sections stained with PAS to assess overall renal structure (A), LTL lectin (red) to evaluate the distribution of PTECs in the kidney (B), and anti-Kim1 antibodies (red) to detect injured PTECs (C). Scale bars 500μm.

Figure 19. Inhibition of RAR by over-expressing dominant negative RAR in PTECs increases tubular injury after IR-AKI

Male *PEPCK-Cre*$^{/+}$; *R26R-RaraDN*$^{+/+}$ (Cre+ PTEC DN RAR) and *PEPCK-Cre*$^{/-}$; *R26R-RaraDN*$^{+/+}$ (Cre- PTEC DN RAR) mice underwent unilateral left renal pedicle clamping followed by delayed contralateral nephrectomy day 8 after the initiating injury. Unless otherwise indicated studies were performed in uninjured Cre- controls (n=3-5), Cre- (n=8-10) and Cre+ (n=4-5) kidneys from mice at day 3 after IR-AKI. (A) Expression of tubular injury marker, *Kim1* mRNA in kidneys day 3 after injury. (B/C) Increased tubular injury in the cortex of PTEC DN RAR mice 3 days after IR-AKI. Representative images (B) and quantification of Kim1 positive surface areas in the OM and cortex 3 days after IR-AKI. (D) Tubular cell proliferation in the OM day 3 after injury. All results expressed as mean +/- SEM. (A) 1-way ANOVA with Tukey's post-hoc correction for multiple-between group comparisons. 1-way ANOVA $p<0.0001$: $^{**}p<0.01$, $^{***}p<0.001$. Comparison with uninjured Cre- controls (no brackets), or between Cre- and Cre+ injured mice (indicated by brackets). (B/D) 2-tailed T-tests, $^{*}p<0.05$.

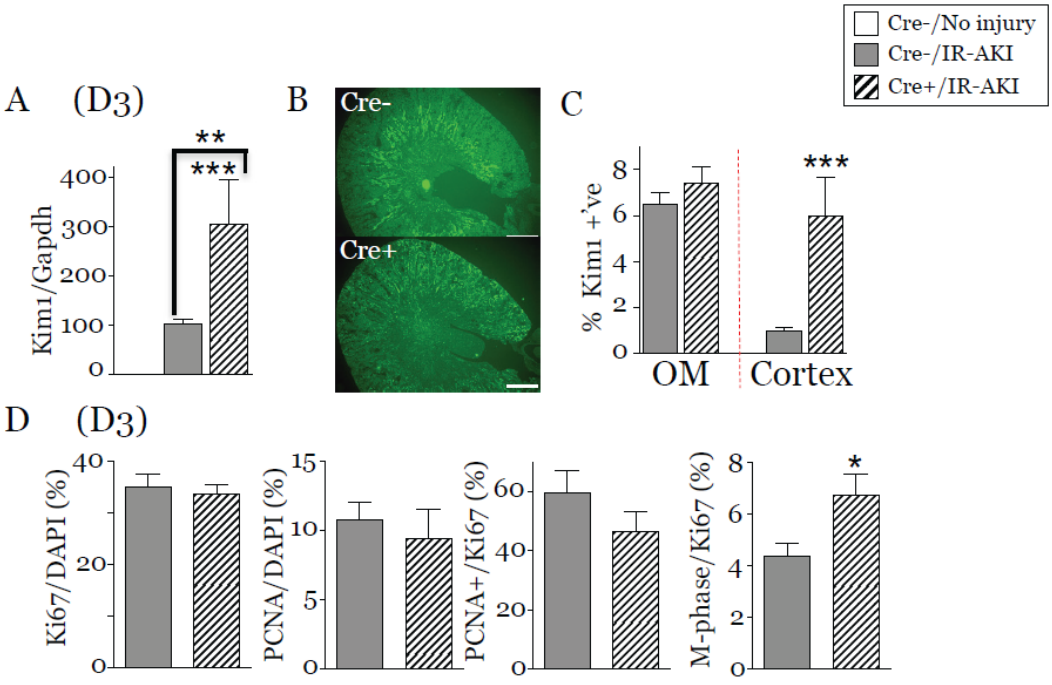
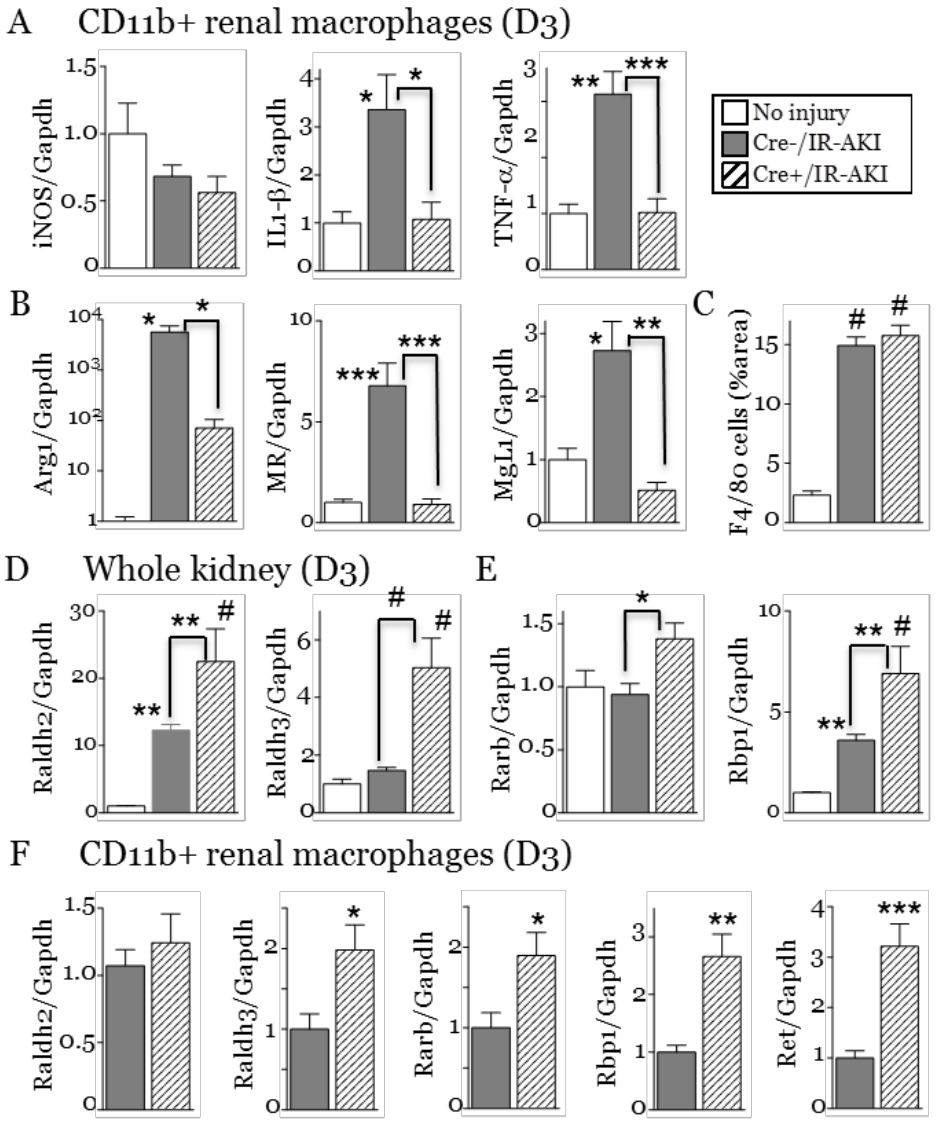

Cre-/No injury
Cre-/IR-AKI
Cre+/IR-AKI
A (D3)
Kim1/Gapdh
400
300
200
100
0
**
***
B
Cre-
Cre+
C
% Kim1 +'ve
8
6
4
2
0
***
OM
Cortex
D (D3)
Ki67/DAPI (%)
40
20
0
PCNA/DAPI (%)
15
10
5
0
PCNA+/Ki67 (%)
60
40
20
0
M-phase/Ki67 (%)
8
6
4
2
0
*

Figure 20. Genetic inhibition of RA signaling in PTECs inhibits M2 macrophage switching after IR-AKI

Male *PEPCK-Cre*$^{+}$;*R26R-RaraDN*$^{+/+}$ (Cre+ PTEC DN RAR) and *PEPCK-Cre*$^{-}$; *R26R-RaraDN*$^{+/+}$ (Cre- PTEC DN RAR) mice underwent left renal pedicle clamping and kidneys harvested 3 days after injury. Studies were performed in Cre- (n=8-10) and Cre+ (n=4-5) mice post-AKI, and uninjured controls (itemized below). (A/B) M1 and M2 markers in renal macrophages. qRT-PCR for M1 (A) and M2 (B) macrophage markers relative to *Gapdh* mRNA control was performed on renal macrophages isolated using CD11b antibody coated magnetic beads. We saw no differences between uninjured Cre- and Cre+ controls, so both control groups were combined for these studies (n=2+3). (C) Renal macrophage numbers, surface area of F4/80-stained macrophages day 3 after injury. Cre- uninjured controls (n=3). (D/E) PTEC-DN-RAR mice have increased expression RA synthesizing enzymes and RA-responsive genes after IR-AKI. qRT-PCR for the RA synthesizing enzymes *Raldh2* and *Raldh3* (D), and RA target genes *Rarb* and *Rbp1* (E) performed on kidneys 3 days after injury. Cre- uninjured controls (n=5). 1-way ANOVA with Tukey's correction for multiple-between group comparisons. Results only indicated if one-way ANOVA $p<0.05$: *$p<0.05$, **$p<0.01$, #$p<0.0001$. Comparison with uninjured controls (no brackets), or between Cre+ uninjured, Cre- and Cre+ injured mice (indicated by brackets). (F) Expression RA synthesizing enzymes and RA-responsive genes in renal macrophages from PTEC-DN-RAR mice after IR-AKI (n=6/group). T-Test: *$p<0.05$, **$p<0.01$. All results expressed as mean +/- SEM, fold change vs.

uninjured controls (A/B/D/E), fold change relative to Cre- injured mouse kidneys (F).



3. Discussion

A signaling is activated and regulates macrophage-dependent injury and repair in the kidney after AKI. Loss and gain of function studies indicate that reactivation of RA signaling does not promote proliferative repair of damaged tubular epithelium in the mouse kidney, but reduces the severity of tubular injury and post-injury fibrosis after AKI. These findings indicate that reactivation of RA signaling is a response to renal injury that limits injury and improves. However, unlike zebrafish larvae, the lack of a growth inhibitory response to blocking RA signaling in mice suggests mouse kidneys may have a reduced regenerative response to RA compared to zebrafish larvae.

Inhibition of RA signaling with BMS493 increases late, post-injury fibrosis but has only limited effects on tubular injury and no effect on renal function after IR-AKI. This contrasts with ATRA, which not only reduces post-injury fibrosis but also accelerates recovery and inhibits tubular injury after IR-AKI. This suggests either that exogenous RA is acting through a mechanism distinct to the intrinsically activated RA signaling pathway in the post-AKI kidney, or that the pharmacological doses of ATRA that we used have more profound effects in suppressing tubular injury than activation of the intrinsic RA signaling pathway.

Macrophage depletion studies show that renal macrophages are required to mediate RA-dependent effects on renal injury. Liposomal clodronate depletes more inflammatory macrophages while alternatively activated macrophages tend to be preserved (Ferenbach et al., 2012). On this basis our data support the hypothesis that BMS493 increases inflammatory macrophage-dependent injury after IR-AKI. Our studies suggest a model in which RA synthesis represses inflammatory macrophages, while activation of RA signaling in PTECs increases

alternatively activated macrophages post-AKI (Fig.21A). The kinetics of RA signaling is consistent with the transition of macrophage phenotypes after IR-AKI (Lee et al., 2011; Zhang et al., 2012). Our model is also consistent with data indicating that: 1) ATRA represses inflammatory cytokine production by cultured macrophages (Mehta et al., 1994; Aggarwal and Mehta, 1996; Dzhagalov et al., 2007; Wang et al., 2007), and 2) PTECs secrete factors that induce expression of alternatively activated markers in cultured macrophages (Alikhan et al., 2011; Zhang et al., 2012; Huen et al., 2014). Our data indicate that RA signaling provides another layer of temporally and spatially controlled signaling that regulates dynamic changes macrophage phenotypes after AKI.

Inhibition of RA signaling in PTECs also inhibits expression of M2 spectrum macrophage markers post-AKI, indicating that there is an RA and PTEC-dependent mechanism regulating activation of macrophages. However, there was also reduced expression of inflammatory macrophages markers in PTEC-DNRAR mice. This may result from a compensatory increase in RA synthesis that in turn suppresses M1 spectrum macrophages in PTEC-DNRAR mice. Depletion of RARα variants in zebrafish embryos also initiates a compensatory increase in RA synthesis (D'Aniello et al., 2013), suggesting that inhibiting RAR signaling activates a similar positive feedback mechanism. On this basis we propose that inhibition of PTEC RA signaling decreases M2 spectrum macrophage markers, but at the same time a compensatory increase in local RA synthesis acts through a different mechanism to repress inflammatory renal macrophages after IR-AKI. Since *in vitro* studies indicate that RA has direct suppressive effects on the expression of inflammatory macrophage markers

(Mehta et al., 1994; Aggarwal and Mehta, 1996; Dzhagalov et al., 2007; Wang et al., 2007), it is likely this is a direct effect of RA on renal macrophages (Fig.10B).

In summary, our results show that RA signaling is activated in mouse and zebrafish kidneys after AKI, and this response limits the extent of injury in both models. These effects are mediated through a previously unrecognized mechanism by which RA coordinates the equilibrium of macrophage activation after AKI. According to this model, repression of inflammatory macrophages by locally synthesized RA reduces macrophage-dependent injury, while locally synthesized RA activates RA signaling in PTECs, which in turn promotes alternative activation of macrophages, enhancing post-AKI repair.

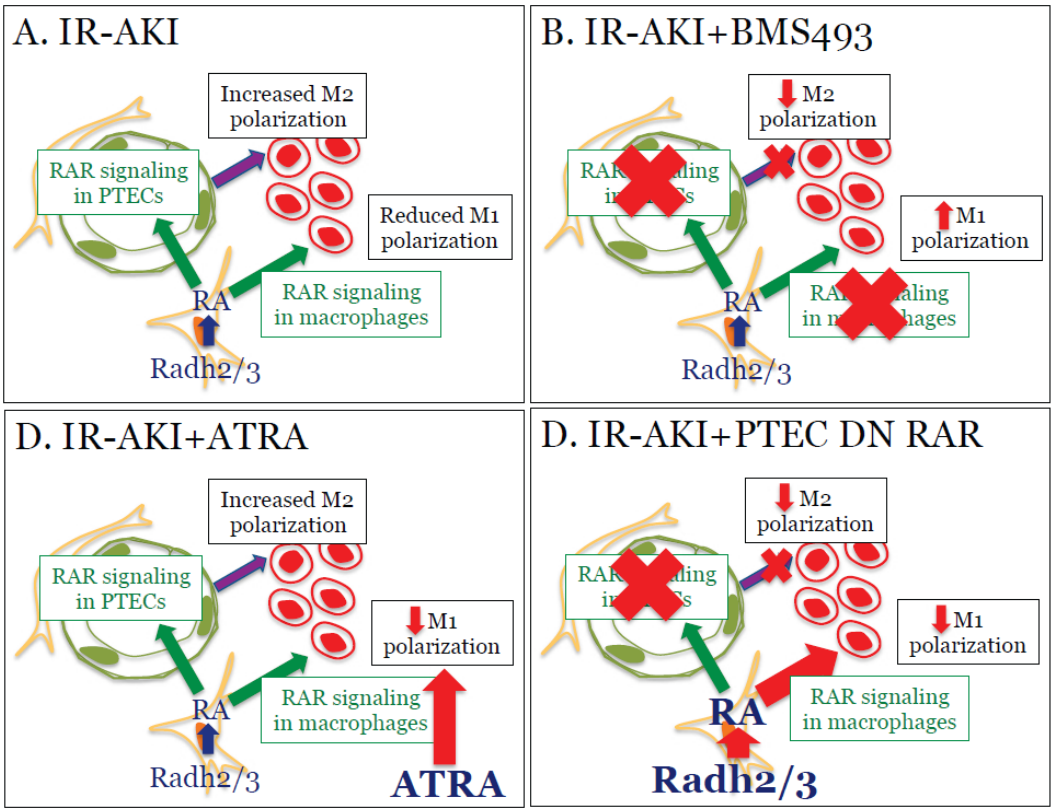


Figure 21. RA signaling regulates renal macrophages phenotypes after IR-AKI

Proposed mechanisms by which RA signaling regulates macrophage polarization after IR-AKI, and how manipulation of RA signaling regulate the balance of M1 and M2 polarization in these studies.

CHAPTER V

Discussion

1. Summary

The primary focus of my dissertation project has been to investigate the role of retinoic acid (RA) signaling in injury and repair using mouse model of ischemia-reperfusion induced acute kidney injury (IR-AKI). Our studies include significant novel findings to expand the field of renal pathophysiology. They also open up many intriguing questions to study, offering ample future opportunities to further understand the normal and abnormal regulation of this process.

I will discuss the importance of our findings in the context of reactivated developmental signaling in tissue repair responses in the first part, and then describe plans future studies to explore new questions raised by this research.

As discussed in *Ch.1 Introduction*, complete resolution of injury is dependent not only on efficient regeneration of damaged tubular epithelium but also on the restoration of tissue oxygenation by damaged microvasculature. These responses are largely determined by the effects of paracrine factors secreted by four dominant cell types; renal tubular epithelial cells, endothelial cells, myofibroblasts and vascular pericytes, and macrophages. While each of these cellular compartments has its origins during kidney development, multiple signaling pathways play important roles to develop each cell types and integrate them into a single kidney. Our fundamental hypothesis was that one of those

developmental signaling pathway is also activated in a cell type and functions to be part of an integrated cellular response that is required for tissue repair after AKI.

RA signaling plays an important role in renal development. The group of Dr. Catherine Mendelsohn demonstrates that; 1) Raldh2 is expressed in cortical stroma and Raldh3 is expressed in ureteric buds (UBs), while expression of both of them starts at E11 stage, and that; 2) Raldh2 plays an essential role in renal development via Ret-dependent branching morphogenesis but Raldh3 plays an auxiliary role for Raldh2 (Rosselot et al., 2010). Key messages from Chapter 1 are there are similarities and dissimilarities between embryonic kidney and reparative kidney in terms of activation of signaling pathways; 1) like development, the pathways are activated after injury in tissues with similar spatial relationship to embryonic origins. Stromal expression of Raldh2 in developing kidneys is reminiscent of what we see expressed in interstitial macrophages in adult kidneys after IR-AKI. This result is also consistent with the recently published TRAP database (Liu et al., 2014). Expression of Raldh3 after IR-AKI is particularly interesting as it is also expressed in infiltrating macrophages, not exclusively in epithelium as in the embryonic kidneys, and it precedes Raldh2 expression. I will discuss the plans for further studies of Raldh3 functions after IR-AKI in Part 3; 2) unlike development, the pathway is activated in more restricted different cell populations or sometimes in different embryonic origins. For example, Raldh2 is expressed in embryonic stroma but is expressed in infiltrating macrophages after injury. RA signaling is activated in UB tips but is activated in reparative PTECs. Redeployment of developmental machinery to

repair process in kidney is a quite interesting phenomena but our study suggests that it is modified with additional regulatory mechanisms.

In addition, we have shown that activation of RA signaling contributes to functional amelioration of post-injury kidney in zebrafish and mouse models, suggesting that the role of RA signaling of embryonic pathways in kidney repair is conserved throughout vertebrate phyla (Chiba et al., 2015a). However, an interesting difference between these two different animal models is RA signaling does acts on promoting proliferation of PTECs in zebrafish but does not in mouse model. This suggests that a different mechanism contributes to post-injury kidney repair in different organisms through which RA singling acts.

While there is no definitive study on the functional role of macrophages in developing kidneys; 1) a study shows macrophages expressing trophic markers are intimately associated with tubular epithelium from the earliest stage of renal development (Rae et al., 2007); 2) its essential roles are demonstrated in many other organs including bone, brain, and retinal vasculatures (Wynn et al., 2013). Macrophages could be an important cell type for kidney development and it would be interesting to analyze RA signaling in macrophages. Our results indicate that injured PTECs are not only a source of progenitor cells for proliferative repair but also a signaling center for macrophages via RA signaling. Injured PTECs act on macrophages and shift them into M2 macrophage spectrum. With the help of those M2 spectrum macrophages, PTECs could complete regenerative repair after IR-AKI.

2. Future Plans

What is the role of RA signaling in macrophages after AKI?

We performed two independent, global and PTEC-specific, loss of function studies of RA signaling after IR-AKI. Our results demonstrate that; 1) inhibition of RA signaling in both, global and PTEC-specific, models inhibits M2 macrophage switching after IR-AKI by using PTEC-DNRAR mice and that; 2) there was also reduced expression of inflammatory macrophages markers in PTEC-DNRAR mice, but not in BMS493 treatment model. These studies revealed that the macrophage is an important cell type in which RA signaling plays a pivotal role in injury and repair after AKI. PTEC-specific RA inhibition study also suggests a compensatory increase in RA synthesis that in turn suppresses M1 spectrum macrophages. It would therefore be interesting to ask; *What is the role of RA signaling in infiltrating macrophages after IR-AKI.* Conditional loss of function study will definitively answer this question. I hypothesize that macrophage-specific inhibition of RA signaling exacerbates injury and phenotypic augmentation of M1 spectrum macrophages after IR-AKI. To test this, I would generate a genetic mouse model, CD11b-Cre; DNRAR, that inhibits RA signaling in macrophages by crossing DNRAR mouse with macrophage specific CD11b-Cre mouse (Boillee et al., 2006). A caveat in use of CD11b-Cre would be that CD11b-Cre; DNRAR could show a problem in macrophage differentiation since CD11b is expressed in all monocytic lineages including macrophages and dendritic cells where RA signaling plays important roles in myeloid differentiation (Nagy et al., 2012) . If this is the case, I would alternatively generate CD11b-Cre-ER^{T} that harbors an inducible Cre driver. I would evaluate

injury, repair, and post-injury fibrosis by analyzing serum creatinine, cell proliferation, cell apoptosis, and expression of Kim-1 and fibrosis markers by using whole kidneys from CD11b-Cre; DNRAR after IR-AKI. I would also evaluate phenotypic switch of macrophages in this model by analyzing markers for M1/M2 macrophage phenotypes with isolated CD11b+ cells by magnetically activated cell sorting (MACS).

I anticipate CD11b-Cre; DNRAR mice show exacerbated kidney injury and/or post-injury fibrosis after IR-AKI since these mice would show further up-regulated M1 macrophage markers. However, it is possible there would be no such anticipated effects in CD11b-Cre; DNRAR mice. If this was the case, there are two possibilities; a) Cre recombination would be inefficient. To determine this, I would isolate CD11b+ cells by MACS from these mice and perform qRT-PCR analysis with RA target genes. If these results confirms RA signaling in macrophages are efficiently inhibited; 2) there would be no direct signaling in macrophages and all effects would be indirect. If so, further studies would explore the role of other cell types (non-epithelial such as endothelial cells, interstitial fibroblasts, and vascular pericytes) in regulating RA dependent macrophages responses.

What is the nature of M1/M2 spectrum switch by RA signaling?

Two key suggestions from our studies are that; 1) RA signaling in macrophages reduces inflammatory macrophage-dependent post-injury fibrosis after IR-AKI; 2) RA signaling activated PTECs mediates augmentation of M2 macrophage spectrum. While we evaluated macrophage M1/M2 phenotypes with selected markers by qRT-PCR, shifts in macrophage phenotypes and activation

can only be determined by comprehensive genome-wide profiling. To do this, I would use two models of RA signaling inhibition that are used in my study; 1) global RA inhibition model; BMS493 treatment and ; 2) PTEC-specific RA inhibition model; PEPCK-Cre; DNRAR. I would induce IR-AKI, isolate CD11b+ macrophages by MACS, and perform RNA-sequencing (RNA-seq) for both models and compare differences between them. I would perform these experiments at day1/3/5 time points. Since Lee et al., 2011 clearly described that expression of a M1 marker, iNOS, peaks at day1 and expression of M2 marker, Arg1, is dramatically up-regulated at day5, we would likely to capture dramatic phenotypic changes of macrophages in these time points. Dr. Lloyd Cantley's group also showed that M1/M2 macrophage phenotypic switch after IR-AKI is different from "canonical" IL-4 induced switch in terms of a M2 maker, Ym1, expression (Lee et al., 2011). It will be intriguing to perform studies that comprehensively characterize infiltrating macrophages in context of post-IR-AKI.

What is the secreting factor (s) from PTECs in macrophage M2 switch?

As summarized in Part1, injured PTECs is an important signaling center that mediates M1/M2 switch of renal macrophages after IR-AKI. Our data indicate that RA signaling provides another layer of temporally and spatially controlled signaling that regulates dynamic changes of renal macrophage phenotypes after IR-AKI. While several studies demonstrate that PTEC-secreted factors CSF1 and GM-CSF plays important roles in M1/M2 switch, these were not regulated in our RA inhibition models after IR-AKI (Zhang et al., 2012; Huen et al., 2014) (data not shown). This suggests that RA signaling- mediated M1/M2

switch is independent from CSF1 and GM-CSF. Therefore, we would identify PTEC secrete factor (s) that mediate M2 macrophage switch under the regulation of RA signaling. We hypothesize that; 1) injured PTECs secrete factor (s) that plays a pivotal role in accelerating macrophage M2 switch and that; 2) RA signaling mediates secretion of the factor from PTECs. To test this, we generated PTEC-specific fluorescent reporter model, PEPCK-Cre; R26R-EYFP (Suppl. Fig XX). By using this reporter model, we isolated YFP+ PTECs with or without BMS493 treatment after IR-AKI at day3 by fluorescently activated cell (FAC)-sorting and would perform RNA-seq. Dr. Skrypnyk in the lab is currently performing this project. With RNA-seq data; 1) I would choose genes that are highly up-regulated plus suggested secretes factors in published literatures after IR-AKI, and are significantly down-regulated with BMS493 treatment; 2) I would perform qRT-PCR experiments for those candidate genes and validate their expression patterns with the same RNA samples. I expect to obtain several candidate genes for further evaluation.

Identification of candidate PTEC secrete factor (s) allows us to characterize its role in M1/M2 switch. First of all, I would use 3 different *in vitro* cultured PTECs models; i) HK-2: immortalized PTECs line from normal adult human kidney (Ryan et al., 1994); ii) Human primary renal PTECs (ATCC PCS -400-010) (Cioni et al., 2013), and iii) immortalized mouse PTECs (Gewin et al., 2012) and test whether these PTECs lines secrete the factors identified above. We would evaluate candidate genes this by; 1) treating with ATRA only and; 2) treating with ATRA with; a) hypoxia-induced injury (Higgins et al., 2007) and; b) antimycin A-induced injury (DiRocco et al., 2014). I anticipate cultured PTECs with injury secrete the factors, but not without injury. ATRA treatment further activates

secretion of the factors from injured PTECs. I also anticipate these responses are conserved throughout mammalian lineages, therefore both human and mouse PTECs lines would show the secreted factors after injury.

I would also use iv) bone marrow derived macrophage (BMDM) cell line and test the effect of candidate PTEC-secreting factor on M2 switch. To do this; 1) we would culture BMDM with supernatant from HK-2 cells after hypoxic injury and with synthetic secreting factor if it is commercially available. We would further evaluate the effects of PTEC secreting factor on BMDM line by loss and gain of function studies; 2) we would perform co-treatment with an inhibitor for the factor if commercially available and/or treat with small interference RNA (siRNA) against mRNA for the factor; 3) we would perform co-treatment with ATRA. I anticipate loss of function experiments show reduced M1/M2 switch and gain of function experiments show augmented M2/M2 switch.

In part of *in vivo* characterization of PTEC secreted factor, I would localize its mRNA by using RNA scope that is highly specific *in situ* hybridization technology (Wang et al., 2012). I expect the mRNA for the secreting factor is detected around its source; PTECs. I would also try to localize its protein by using its antibody if it is commercially available and confirm its expression and interaction with macrophages. Successful localization of PTEC secreted factor is important because it allows us to study its function in detail by an *in vitro* cell culture model and an *in vivo* cell-type specific loss of function mouse model. For the mouse model, we could use a floxed mouse line for the PTEC secreted factor and cross with PEPCK-Cre to induce PTEC-specific knockout for the secreted factor. I anticipate the inhibition models of the factor secretion from PTECs unable to show M1/M2 switch in macrophages.

What is the role of Raldh3 in post-AKI repair?

We show that dynamic expression of Raldh1-3 after IR-AKI. Of those, Raldh3 is the earliest Raldh isoform that is up-regulated after IR-AKI. The kinetics of Raldh3 synchronizes with its RARE-hsp68-lacZ reporter, suggesting that Raldh3 is the important enzyme among Raldh isoforms. We also showed that Raldh3 expression is localized to early infiltrating macrophages after IR-AKI. I therefore hypothesize that macrophage specific knockout of Raldh3 exacerbates injury and/or post-injury fibrosis after IR-AKI. To test this, we would generate CD11b-Cre; Raldh3$^{flox/flox}$ mouse by obtaining ES cells for Raldh3$^{flox/flox}$ from UC Davis Knockout Mouse Project (KOMP) Repository. In case CD11b-Cre; Raldh3$^{flox/flox}$ show a problem in macrophage differentiation, I would alternatively generate CD11b-Cre-ER^{T} as discussed in the plan for CD11b-Cr; DNRAR mouse.

By using CD11b-Cre; Raldh3$^{flox/flox}$ mouse model, I would; i) evaluate injury, repair, and post-injury fibrosis by analyzing serum creatinine, cell proliferation, cell apoptosis, and expression of Kim-1 and fibrosis markers by using whole kidneys from CD11b-Cre; Raldh3$^{flox/flox}$ after IR-AKI; ii) also cross CD11b-Cre; Raldh3$^{flox/flox}$ with RARE-hsp68-lacZ reporter and test whether macrophage-specific knockout of Raldh3 significantly down-regulates RARE reporter expression; iii) perform FACS analysis to evaluate Raldh activity in macrophages from CD11b-Cre; Raldh3$^{flox/flox}$ by gating CD45/CD11b/F4-80/Aldefluore; iv) finally isolate macrophages by CD11b+ MACS and evaluate macrophage phenotypes by qRT-PCR using M1/M2 macrophage markers.

3. Concluding remarks

The studies presented here demonstrate that RA signaling is dynamically activated in the kidneys after IR-AKI. We identified macrophages is an important cell type where RA signaling acts on. Proper regulation of macrophages by RA signaling coordinates injury and repair, and is required for reducing post-injury fibrosis. While we do not yet understand upstream mechanism or downstream effector for RA signaling, this work has opened other avenues worthy of investigation which should shed light broader aspects of kidney injury and repair. Most notably, we have established two independent, global and PTEC-specific, loss of function models of RA signaling. We identified the novel mechanisms in which; 1) RA acts directly on macrophages to reduce its inflammatory phenotype and; 2) the interaction of PTECs and macrophages via RA signaling. These models can be powerful tools that can be further dissects the function of RA signaling in the setting of IR-AKI.

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