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Th-17 cell activation in response to high salt following acute kidney injury is associated with progressive fibrosis and attenuated by AT-1R antagonism

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Abstract

Exposure of rats to elevated dietary salt following recovery from acute kidney injury (AKI) accelerates the transition to chronic kidney disease (CKD), and is dependent on lymphocyte activity. Here we tested whether high salt diet triggers lymphocyte activation in post-ischemic kidneys to worsen renal inflammation and fibrosis. Male Sprague- Dawley rats on a 0.4% salt diet were subjected to left unilateral ischemia-reperfusion and allowed to recover for 5 weeks. This resulted in a mild elevation of $CD4^+$ T-cells relative to sham animals. Contralateral unilateral nephrectomy and elevated dietary salt (4%) for 4 extra weeks hastened CKD and interstitial fibrosis. Activated T cells were increased in the kidney 3-fold after 4 weeks of elevated dietary salt exposure relative to post AKI rats prior to salt feeding. The T-cell subset was largely positive for IL-17, indicative of Th-17 cells. Because angiotensin II activity may influence lymphocyte activation, injured rats were given the AT1R antagonist, Losartan, along with high salt diet. This significantly reduced the number of renal Th-17 cells to levels of sham rats, and significantly reduced the salt-induced increase in fibrosis about half. *In vitro* studies in AKI-primed $CD4^+$ T cells indicated angiotensin II and extracellular sodium enhanced, and Losartan inhibited IL-17 expression. Thus, dietary salt modulates immune cell activity in post ischemic recovering kidneys due to the activity of local RAS suggesting participation of these cells in CKD progression post AKI.

Keywords

Acute kidney injury; renin angiotensin system; chronic kidney disease; ischemia reperfusion; lymphocytes

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INTRODUCTION

Acute kidney injury (AKI) secondary to ischemia or nephrotoxicity is characterized by rapid decline of GFR, decreased blood flow and increased local and systemic inflammation leading to tubular damage[1]. Clinically, AKI is also associated with prolonged stay in the hospital and high mortality rate. Although recovery of renal function is frequently observed in survivors, recent data indicate that a significant proportion of patients progress to CKD and ESRD following AKI[2]. In animal models of AKI, resolution of kidney function and repair of the tubular epithelium occur fairly rapidly. However, repair is often not complete and modest but persistent changes in the kidney structure predispose it towards chronic injury disease (CKD)[3-7]. In the rat model of I/R induced AKI, high salt diet starting 5 weeks after of recovery accelerates the AKI-CKD transition, which is characterized by fibrosis, proteinuria, hypertension and impaired hemodynamic responses[3]. The basis of progression of CKD in AKI rats fed high salt diet is not clear.

One of the primary responses to any insult is the production or release of proinflammatory proteins, which act as immune modulators. Previous studies from our laboratory observed that activated CD4+T cells persist in the kidney even up to 5 weeks of recovery[8] suggesting that prolonged T-cell exposure could participate in the AKI to CKD transition. This concept is strengthened by the demonstration that treatment with mycophenolate mofetil (MMF), an inhibitor of T cell activation, blocked the proteinuria, fibrosis and hypertension in post ischemic rats fed on high dietary salt[9].

Following ischemia and reperfusion, the renal parenchymal cells are injured and they play a critical role in facilitating the trafficking of innate lymphoid cells like dendritic cells and macrophages into the kidney[10]. These antigen-presenting cells provide activating signals for T and B cells to migrate to the site of inflammation. T cells, depending upon the activating signals, can differentiate into different effector T helper subsets. It was initially thought that Th1 cells, characterized by IFN-γ secretion, promoted pathogenesis of AKI whereas IL-4 secreting Th2 cells protected from AKI. But recent data, suggest that Th17 cells play a role in tissue injury as IL-17A deficient mice (fewer Th17 cells) manifest reduced severity of acute injury in response to cisplatin [11], and recently, Th17 cells were implicated in the inflammation following renal ureteral obstruction [12]. In addition, infusion of IL-17 into the lung exacerbates smoke induced fibrosis[13]. Based on these observations, it is important to identify specific T-helper populations following tissue injury to further delineate the role of T helper subsets in AKI-CKD transition as well as during CKD.

The Renin Angiotensin system (RAS) is thought to play a significant contributory role in the progression of CKD and hypertension. Ang II, the main mediator of RAS pathway, promotes vasoconstriction, fibrosis, apoptosis, oxidative stress and inflammatory processes, all of which are implicated in kidney injury. Various studies involving genetically modified mice as well as treatment with pharmacological inhibitors have established the importance of Ang II in chronic kidney disease[14-17]. Ang II acts as accessory signal for T cell function. Silva-Filho et al., confirmed that T cell receptor ligation increases AT1R expression[18]. Ang II acts as a chemoattractant for T cells to the site of inflammation and *in vitro* studies indicate that Ang II also behaves as a costimulator for T cell activation[19]. In addition,

lymphocytes, in particular Th17 cells, have been implicated in the full manifestation of increased blood pressure in response to Ang II infusion in mice [20, 21]. Conversely, inhibition of the RAS by losartan or captopril reduces T cell proliferation, adhesion and chemotaxis in mice infected with *Plasmodium berhei* [18].

The following study was therefore conducted to evaluate the effect of high salt diet and the role of AT1R receptor activity on CD4 T cell activation and the potential effects on the progression of CKD following recovery from AKI.

RESULTS

Lymphocytes influence the progression of AKI to CKD

To evaluate role of lymphocytes as a function of transition following repair to CKD progression, rats were subjected to unilateral I/R and allowed to recover for 35 days. Some rats were then subjected to unilateral nephrectomy and exposed to elevated salt diet for additional 4 weeks (to *day 63* post I/R), which resulted in proteinuria and progressive fibrosis similar to previous studies (data not shown). A robust increase in infiltration of immune cells specifically CD4+T cells, CD8+T cells, B cells and macrophages/DC was observed in the injured kidney as compared to sham-operated rats 35 days post-surgery (Figure 1). Elevated salt diet significantly and markedly increased CD4+ T cells by ~ 3.5 fold in the injured kidney but did not influence CD4+ T cells in kidneys of sham rats (Figure 1A). High salt diet did not further enhance the CD8+ T cells or B cells in directly injured kidneys but high salt did increase B cells in contralateral kidneys (Figure 1B and C). The number of DC/macrophages were elevated following injury but unexpectedly resolved to sham levels in response to high salt diet. (Figure 1D).

The activation marker CD25 was moderately enhanced in T cells from kidney 35 days following recovery from I/R injury and this activation was prominently enhanced by subsequent exposure to high salt diet (Figure 1E). T-cell CD25 expression was also increased in response to high salt diet in contralateral kidneys, but not kidneys of sham rats. Interestingly, CD4+T cells isolated from the kidney predominantly secreted IL-17, indicating that these cells are skewed towards Th17 phenotype (Figure 1F). High dietary salt also increased IFN-γ secreting (Th1) and IL-4 secreting (Th2) CD4+ T cells, but the proportion of these T cells was much lower than the IL-17+ T cells (Figure 1F). Figure 1G illustrates the amount of renal Th17 cells at various times following I/R injury. At day 1 and 3 post I/R (when damage is typically severe) CD4+/IL17+ T cells were dramatically enhanced in the direct injured and to a lesser extent in the contralateral kidney relative to sham. Th17 cell expression resolved, albeit non-completely in injured kidney as the animals entered the recovery phase (i.e., between *day 7 to 35*), but increased in response to high salt diet in both the injured and contralateral kidney (Figure 1G).

We analyzed the mRNA expression of cytokines and transcriptional factors associated with T helper differentiation either in whole kidneys tissue or isolated CD4+T cells. Surprisingly we did not detect mRNA expression of IL-17 cytokine in either whole kidney or isolated cells (not shown), however the mRNA expression of the transcription factor RORγC, a regulator of Th-17 differentiation, was significantly elevated in the T cells isolated from the

directly injured kidney relative to contralateral kidney (Figure 2A), and was not detectable in cells from sham-controls (not shown). Interestingly the expression of RORγC mRNA was higher in the whole tissue relative to isolated T cells, suggesting that kidney resident cells also express RORγC (Figure 2A). The mRNA expression of cytokines IFN-γ and IL-4 were significantly higher in CD4+T cells isolated from injured kidney vs contralateral (Figure 2B and 2C) and neither were detected in cells from shams. Furthermore, the mRNA for Tb×21, a transcription factor associated with Th-1 differentiation, was also elevated in isolated cells from injured vs contralateral kidney (Figure 2D).

Influence of AT_1R activity on lymphocytes post AKI

Ang II sensitivity is increased post AKI and high salt diet enhances Ang II induced tissue damage [1, 22]. To determine if Ang II activity would influence T cell activation during AKI to CKD progression, rats were subjected to unilateral I/R and subsequent unilateral nephrectomy of the contralateral kidney at *day 35*. Rats were then treated with high salt diet with or without losartan (an AT_1R antagonist, 30 mg • kg^{-1}•day^{-1}), for 4 weeks (Day 35-63 post surgery). As expected, an increase in interstitial fibrosis (by picrosirius red; Figure 3A and B) and an increase in interstitial cell volume (Figure 3C and D) were observed in post ischemic kidneys relative to sham and these parameters were significantly attenuated by losartan treatment. At the termination of the experiment, total renal blood flow, measured under isoflurane anesthesia, was significantly reduced in post AKI rats vs. sham, while losartan restored RBF values in sham rats (Figure 3E). Baseline blood pressures under anesthesia were not different between groups (not shown). Despite the effects on tissue morphology and RBF, losartan failed to restore GFR as measured by 24-hour creatinine clearance (Figure 3G), nor did it protect against the increase in albumin excretion induced by high salt in post ischemic rats (Figure 3F).

Losartan treatment attenuated the total CD4+T infiltration in post ischemic rat kidneys (Figure 4A), but did not have a significant effect on the on percentage of the CD25 or CD62L expression on T cells as compared to post IRI rats. (Figure 4B). However, losartan significantly reduced the increase in expression of CD4+/IL-17+ to levels observed in sham controls (Figure 4C). Losartan also reduced the IFN-γ positive CD4 cells (Figure 4D) as well as the number of IL-4 positive cells (Figure 4E).

It is unclear whether normalization of Th17 cells by losartan is the result of direct effects on T cell activation or occurs secondary to a generalized protection against CKD development due multiple effects of AT_1R antagonism. To determine if Ang II may directly influence T cell differentiation via AT_1R activation, it was first determined whether AT_1a and AT_1b receptor mRNAs were expressed in renal T cells. Both of these mRNAs were expressed at higher levels in T cells, relative to whole kidney tissue, although these levels were not influenced by injury (Figure 5A and 5B).

Next, T cells were isolated from AKI and sham operated rats 7 days post surgery and incubated *in vitro* with varying concentrations of NaCl, Ang II and losartan overnight. Increasing the extracellular Na^+ concentration (from140 mM to 170 mM) resulted in a small but significant increase in IL-17 mRNA expression in post-AKI T cells but not sham primed T cells (Figure 5C). Although Ang II had little effect on IL-17 mRNA in T cells under

standard Na^+ conditions, the IL-17 mRNA response was synergistically enhanced with elevated extracellular Na^+. This response was observed only in T cells isolated from injured rats, and was completely blocked by losartan (Figure 5C). Similar regulation of IL-17 mRNA by Ang II and increased Na was measured in T cells isolated 2 days post-surgery (data not shown). Message levels of IFN-γ were unaffected by treatments (Supplemental Figure 2) and IL-4 mRNA was undetectable (not shown). Taken together, the data suggest that AT_1R activity may directly regulate T cell stimulated IL-17 production following kidney injury.

DISCUSSION

Acute kidney injury predisposes the development of chronic kidney disease, however the mechanism of this transition is unclear. Studies in animal models of AKI suggest reduced capillary density, hypoxia, oxidant stress, increased Ang II sensitivity, activation of profibrotic factors by interstitial cells or tubular epithelial cells, or activation of inflammatory/immune mediators may participate in the progression of fibrosis following acute kidney injury [5, 6, 23-2728, 29]. Pathophysiological variables introduced into animal models have been used to investigate such mechanisms in more detail. For example, reductions in renal mass accelerate progression following AKI, which may be due to exacerbated hypoxia from reduced capillary density [24], an increased inflammatory environment resulting from failed recovery of tubular epithelial cells [5, 26] and/or enhanced degree of hypertension [26]. In addition, we have also shown that exposure to increased dietary salt (to 4%) following 5 weeks of recovery from AKI resulted in hypertension and rapid progression CKD in rats post I/R injury [28]. The cause of hypertension resulting from high-salt diet may be due to impairment of renal hemodynamic responses [30]. The enhanced progression of CKD may also be due to inflammation secondary to renal lymphocyte activity following exposure to elevated dietary salt [23, 31].

In the current study, we provide evidence that post ischemic rats fed on high salt diet displayed a local Th17 inflammatory response. We observed a biphasic response of Th17 infiltration; during acute kidney, when animals are on normal salt diet, and then again following repair from I/R when rats were placed on high salt diet to hasten CKD. These results suggest that Th17 activation may influence both early injury events as well as events leading toward progression of chronic disease. Other immune cells such as B cells, CD8+T cells and innate cells were also induced by I/R but their numbers were unaffected by high salt diet suggesting their role is primarily related to early ischemic damage rather than secondary development of chronic disease. Further, administration of losartan protected against the development of fibrosis and preserved renal blood flow during AKI to CKD progression. Losartan also reduced activation and differentiation of CD4+ T cells to Th17 cell lineage *in vivo*, suggesting that AT_1R activity is enhanced by high salt feeding and may modulate progression post ischemia by affecting lymphocytes.

Renal fibrosis is a common feature of CKD irrespective of the initial cause. During progressive disease, there is increased tissue scarring leading to the loss of tissue parenchyma and further reducing renal function [32]. The underlying mechanisms of renal fibrosis following acute ischemic injury is complex, but involve an expansion of the

interstitial space with activation of pericytes[33]. Infiltrating immune cells also contribute to fibrosis. Macrophages produce profibrotic and proinflammatory factors that help in the maintenance of mesenchymal cells thereby promoting fibrosis. T cells also promote fibrosis by recruiting neutrophils to the site of injury. Studies in humans have confirmed that neutrophil to lymphocyte ratio was good indicator for CKD progression[34].

T cell activity has previously been implicated in ischemia/reperfusion injury of various organs[35, 36]. Burne-Taney et al., demonstrated that $CD4^{-/-}$ mice were protected from renal I/R injury, while adoptive transfer of wild-type CD+ T cells restored I/R injury confirming the importance of these cells in the genesis of AKI[37]. Additional studies by multiple groups have suggested that the STAT6/IL4 pathway in Th2 cells is protective in AKI and enhances repair, while the STAT4/IFN-γ pathway of Th1 cells plays a modest contributory role toward inflammation in I/R induced AKI [383940]. Recently, a new proinflammatory T helper subset, referred to as the Th17 cell, has been associated with various immune disorders [41-43]. It has recently reported that there is infiltration of Th17 cells following acute ureteral obstruction [44]. Interestingly, RORγC null mice, which have fewer Th-17 cells, were significantly protected from cistplatin induced AKI[11]. In the current study, significant Th17 cell activation was observed within 1-3 days of I/R injury in rats, however since Th17 cells were dramatically increased in both the injured as well as non-injured contralateral kidney, the massive influx of Th-17 cells is not sufficient, by itself, to drive acute injury. Regulatory T cells, (Tregs), suppress the activity of Th1/2 and Th17 cells. Kinsey et al., have shown that partial depletion of these cells potentiated acute kidney damage in mice [45]. In unpublished studies from our lab, we have verified that renal Tregs are also increased at early time points post ischemia in rats (i.e., 1-3 days, not shown) but how high salt diet and Ang II influence renal Treg activity chronically post AKI has not yet been evaluated.

While lymphocytes have been investigated during the acute response to I/R, thus far, little effort has been directed toward investigating their role in CKD following AKI, with notable exceptions. Burne-Taney et al., demonstrated that adoptive transfer of lymphocytes from post ischemic mice into naïve mice resulted in proteinuria in the recipients[46]. Previous studies from our laboratory indicate that immune cells persist in kidneys following recovery from renal I/R at least up to 5 weeks following recovery [31] and that immune suppression with MMF attenuated renal fibrosis and hypertension in post AKI rats fed a high salt diet [47]. However, the specific cell types mediating progression in response to high salt diet post AKI has not been appreciated.

The current results indicate that dietary salt enhances the Th17 response following recovery of the kidney to I/R injury, suggesting that this cell type may be a central mediator to the secondary inflammation and fibrosis, which occur in this setting. To our best knowledge, no prior study has described Th17 cells in the AKI to CKD progression. A potential role of Th17 cells in renal fibrosis is consistent with reports using other models of CKD. For example, transgenic mice lacking IL-17 cytokine or Th17 cells, displayed reduced development nephrotoxic induced glomerular nephritis[48]. Peng et al., showed that IL-17A from γδ and a CD4+ T cell significantly contributes to renal fibrosis by regulating chemokine RANTES during obstructive renal injury [49]. Th17 cells were detected in the glomeruli and tubulointerstitium of ANCA associated glomerulonephritis in patient renal

biopsies[50]. Th17 cell differentiation depends upon two pleitrophic cytokines IL-6 and TGF-β. Increased levels of both the cytokines in response to injury have been measured in multiple studies. Further administration of IL-6 exacerbated AKI induced fibrosis[51].

Based on these reports and the prominent induction of Th-17 cells in response to elevated dietary sodium report here, we suggest that Th17 cells may participate as a pro-inflammatory and fibrotic mediator of the AKI to CKD transition. However, the evaluation of this hypothesis requires a much more direct investigation than we have utilized in the current study. Indeed, other lymphocytes such as Th-1 and Th-2 might play a significant pro-fibrotic role despite their relatively lower response to high salt diet. In addition, the inhibition of Th17 cells by losartan occurs concurrently with an inhibition of Th-1 and Th-2 cells, all of which may be primary or secondary activities in protection shown in this study. Future studies will be directed toward evaluating these cell types in greater detail.

The immediate increase in Th-17 cells during the early phase of injury and repair is not associated with the development of interstitial fibrosis in either the contralateral or injured kidney, since recovery up to 5 weeks is not associated with significant scarring. Moreover, it remains to be determined if the later increase in Th-17 cell infiltration plays a functional role in CKD or is elevated as a consequence of CKD. If Th-17 cells are profibrotic, the lack of early fibrosis in our model may rely on the degree to which Th17 cells are sustained post injury, being reduced to control levels within 7 days of recovery. In contrast, the secondary activation of this pathway by high salt diet results in a steady increase in the presence of Th17 cells representing a potential sustained pro-fibrotic effect. Similarly, TGF–β1, a prominent profibrotic factor, is increased for up to 2 weeks post I/R but returns to baseline expression within 4 weeks in rats without significant fibrosis. However there is a re-expression of TGF–β1 as progressive CKD develops with longer recovery [52, 53].

While dietary salt increases the activity of renal lymphocytes post AKI, the mechanism of how this occurs remains unclear. Lymphocytes have been implicated in the degree to which salt sensitive hypertension and inflammation are manifested in models such as the Dahl S salt sensitive rat, a low renin model of hypertension. Interestingly, Dahl S rats on high salt are protected from injury by Ang II antagonism [54]. De Miguel et al., demonstrated that while circulating renin and Ang II are repressed in DahlS rats on high salt, renal levels of Ang II are not repressed by high salt [55]. Furthermore, these investigators demonstrated a high expression of renin-angiotensin components within renal lymphocytes relative to whole kidney tissue [55], suggesting that the local rather than circulating RAS activity may participate in salt-induced lymphocyte activation.

The mechanism by which high salt diet or Ang II can directly influence lymphocyte activity in vivo is unclear. Recent data suggest that interstitial Na concentration may be more variable than historically appreciated and in vitro studies have shown that varying extracellular sodium can induce T cell differentiation toward a Th17 phenotype [56]. Moreover, infusion of Ang II in mice increased IL-17 secretion from aortic T cells [21]. Our data indicate that IL17 mRNA expression is synergistically activated by Ang II in the presence of increased extracellular Na+ (to 170 mM). Interestingly we observed an increase IL-17 expression only in AKI-primed T cells but not in T cells of sham-operated rats. In

activated Th17 cells, the IL-17 gene is highly methylated or open for transcription whereas naïve T cells are hypomethylated [57]. Furthermore, our studies indicated that ischemia/reperfusion increased the number of activated T cells, hence proportion of activated T cells with an open IL-17 reading frame may be higher in AKI-primed T cells as compared to sham operated T cells. Thus we propose that sustained renal Ang II, in combination with elevated sodium, represents a potential activating stimulus for lymphocytes which may be epigenetically primed following kidney injury. While it is interesting that such a synergistic effect on lymphocytes exists, it must be pointed out that levels of renal Ang II and levels of renal interstitial sodium have not been measured in post AKI rats in response on low or high salt diet and therefore, we cannot be certain that dietary salt influences the local milieu in such a way as to induce T cell differentiation by this mechanism.

In summary, we have observed that Th17 cells are associated with the salt-induced progression of AKI to CKD, in an AT_1R dependent fashion. These cells represent the most prominent cell type activated by high-salt diet in the post ischemic kidney. Since these cells are pro-inflammatory and also associated with hypertension, they represent a potential important cell type mediating AKI to CKD. While AT1R activity appears important in Th17 cell activation, losartan is well known to influence multiple other activities, which may influence progression post AKI, such as increasing renal blood flow or inhibiting pro-fibrotic factors such as TGF-β. Therefore, future studies will be required to determine whether Th17 cell suppression plays a primary role in the mediation of CKD following recovery from AKI.

METHODS

Animals

Male Sprague-Dawley rats (250–300g) were purchased from Harlan (Indianapolis, IN) and used for all studies. Rats were maintained in accordance with the policies of the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. All protocols were approved by Institutional Animal Care and Use Committees at Indiana University.

Study protocols

Study 1 was designed to evaluate the lymphocyte characterization and differentiation following AKI and progression to CKD. Rats were subjected to unilateral (left) ischemia reperfusion (I/R) injury or sham surgery (see below) and allowed to recover for various times following surgery for 35 days. At the indicated times, both kidneys (injured and contralateral) were analyzed for lymphocyte expression. Some rats were subjected to unilateral nephrectomy of either the injured or contralateral kidney at 35 days following recovery and were subsequently exposed to elevated dietary salt (4% NaCl) on the following day. As described in our previous report[8], removal of the contralateral kidney and exposure to high-salt diet, hastened the development of CKD, while removal of the injured kidney leaving the contralateral kidney intact results in hypertension, impaired renal hemodynamic responses, but a less severe fibrotic renal injury[8]. Kidneys from direct injury, contralateral or sham groups were harvested at 63 days (following 4 weeks of high salt diet treatment) and utilized for analysis of lymphocyte expression.

Study 2 was designed to study the effect of Ang II receptor (AT_1R) blockade on the AKI to CKD progression. This study was performed similar to study 1 except at the time of unilateral nephrectomy (35 days), only contralateral kidneys were removed to evaluate CKD development in the injured kidney. The following day, when rats were placed on high salt diet, post AKI rats were maintained on either normal drinking water or water supplemented with losartan to achieve a target does of 30 mg • kg-1• day-1.

Measurements of renal functions

Blood from rats was collected via tail clipping in heparinized tubes and spun to collect plasma. Urine collections were carried out at multiple times post surgery for 24 hours by placing rats in the metabolic cages and urine volume was determined gravimetrically. Serum creatinine was measured with a Pointe Scientific creatinine kit and QT 180 analyzer (Pointe Scientific, Canton, MI). Urine creatinine was measured using a similar colorimetric assay adopted for microplate readers, as previously described [52]. Creatinine clearance was measured using U_{Cr}* (urine flow rate)/P_{Cr} and values normalized to body weight. Urine albumin levels were measured using albumin blue 580 fluorescence method as described previously [58]. Data is expressed as total daily albumin excretion normalized per 100g body weight.

Renal blood flow measurements

In some studies, renal blood flow was assessed at the completion of the study and just prior to euthanasia. Rats were anesthetized and prepared for renal blood flow (RBF) analysis as described previously [59]. Briefly, a midline abdominal incision was made, and a flow probe was placed around the renal artery for measurement of RBF via an ultrasonic Doppler flowmeter (model TS420, Transonic Systems, Ithaca, NY). Following a 30-min equilibration period, RBF data were collected and summarized from the final 10 min of stable measurements.

Renal histology and immunohistochemistry

At the end of either study, rats were deeply anesthetized by pentobarbital and kidney tissue harvested for subsequent analysis. Care was taken to bisect the kidney so that proportional degrees of cortex and medulla were represented. Formalin-fixed tissues were embedded in paraffin and stained with picosirus red to assess fibrosis and PAS to calculate interstitial space. For quantitative analysis, five random images of the outer medulla per kidney were captured. The % area of the picrosirus red stain was calculated with the help of Image J as described previously[22].

FACS analysis

Harvested kidneys were minced and digested in liberase (2μg/ml; Roche) for 15 min at 37°C with the help of Gentle MACs (Miltenyli). The digested tissue was filtered through a 100-μm filter mesh and washed with tissue culture medium. The lymphocytes were separated by Percoll (Sigma) and counted by hemocytometer. To evaluate T lymphocytes, the cells were stained with antibodies against rat CD4 (PE-Cy7), CD8a (Alexa 647), CD25 (FITC), and CD62L (PE). To evaluate the cytokines secreted by T cells, the cells were stained for CD4

surface marker and then permeabilized using saponin and stained with antibodies against rat IFN-γ (FITC), IL-4 (PE), IL-17 (FITC) and TNF-α (PE). B cells were stained with antibody against RTIB (FITC) and macrophages were stained using anti-CD103 (PE). All antibodies were obtained from BD Bioscences. Cells were scanned using flow cytometry (FACSCalibur, BD Biosciences) and scans were analyzed using Flowjo software (Tree Star, Ashland, OR). The lymphocyte gating strategy used was described in our previous report[8] and is diagrammed in Supplemental Figure 1. The data is expressed a total number of specific cell population per gram of kidney.

In vitro T cell stimulations studies

Kidneys were harvested from rats on day 7 following ischemia/ reperfusion. Mononuclear cells were separated using Percol according to the protocol described above. CD4+T cells were isolated using Macs separation kits (Miltenyl). T cells were stimulated with anti-CD3 (2μg/mL) and anti-CD28 (1μg/mL). Cells were treated with elevated Na^+ (170mM), Ang II (10^{-7}M) and losartan (1μM) or a combination of these overnight.

RNA analysis

Total RNA was obtained from kidney or isolated cells using Trizol and Zymogen RNA extraction kit and cDNA was prepared using MMLV enzyme. Quantitative real time using gene specific primers (Life technologies; supplemental Table 1) was performed using 7500 ABI biosystem machine. Relative expression was calculated using Ct value.

Statistical analysis

All data are expressed as means ± SE. Differences in means were established by Student's *t*-test

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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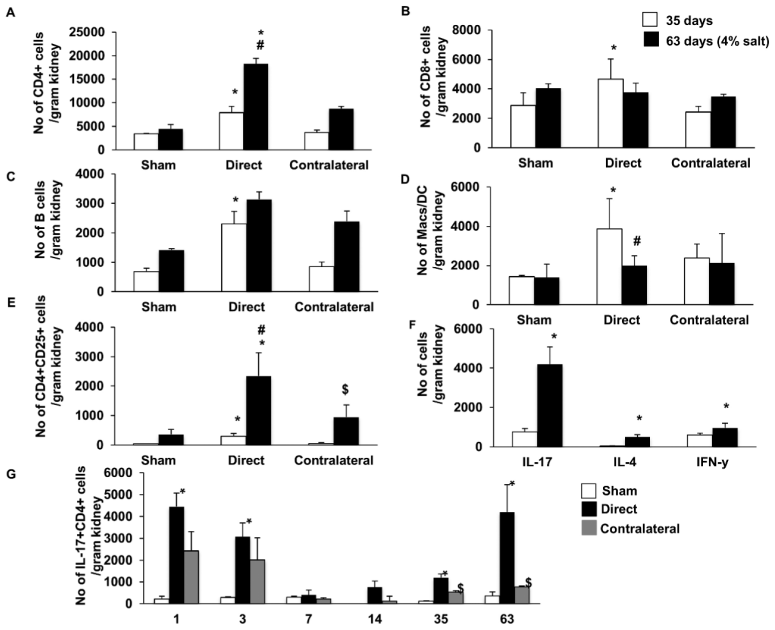

Figure 1. Phenotype of kidney lymphocytes in post ischemic rats

Lymphocytes were obtained from rats following renal I/R injury or sham surgery (35 days) and after an additional 4 wk on a high-salt (4.0% NaCl) diet (63 days) and subjected to flow analysis to identify specific populations in response to injury. The number of CD4+ (A), CD8+ (B), B cells (C), DC/Macs (D) and CD25+CD4+ (E) isolated from injured (direct), contralateral or sham kidney are shown. Panel F illustrates quantitative analysis of IL-17, IL-4 and IFN-γ stained CD4+T cells from post ischemic and sham rats fed on high salt diet at 63 day in injured kidney. (G) Temporal changes in the number of IL-17 T cells are shown for injured, sham and contralateral kidney at various times post-surgery. Data are mean ±SEM; * indicates $P < 0.05$ vs. sham group, $ indicates $P < 0.05$ contralateral vs. direct injury (n=3-5 animals/group).

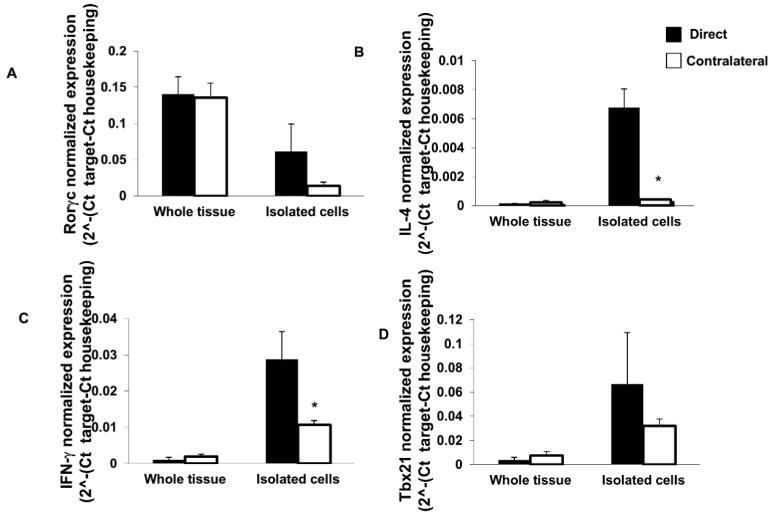

Figure 2. Expression of mRNA for cytokines and transcription factors associated with T helper cells in post ischemic rats

Messenger RNA expression of transcription factors RORγC (A) and Tb×21 (D) and cytokines IFN-γ (B) and IL-4 (C) in injured vs contralateral kidney are shown for whole kidney tissue and isolated T cells 63 days post-surgery. Data are mean±SEM. * indicates P> 0.05 direct vs. contralateral (n=4 rats/group)

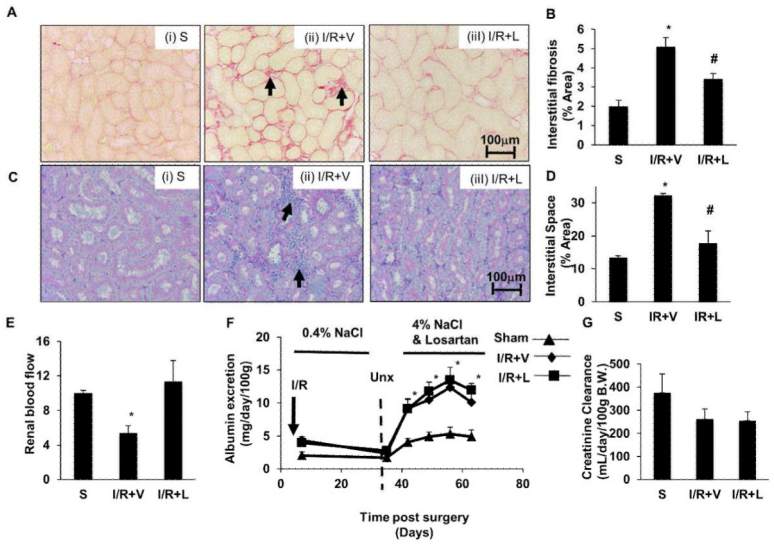

Figure 3. Effect of losartan on renal structure and function in post ischemic and sham-operated rats fed on high salt diet

Representative images through the renal outer medulla are shown for (A) picosirius red and (B) PAS stained sections of kidneys from (i) Sham+high salt diet (ii) IR+Vehicle+high salt diet and (iii) IR+losartan+high salt diet rats. Increase in interstitial fibrosis and tubulointerstitial space is apparent in vehicle treated rats post IR, which was reduced by losartan treatment. Magnification is shown in iii. (C) Quantification of interstitial fibrosis area based on the picosirius red staining area is shown. (D) Quantification of interstitial space is shown, based on the PAS derived by counting the number of points in the arbitrary grid that overlay the interstitial space. (E) Total renal blood flow by transit time ultrasound measurement through the renal artery is shown. (F) Daily albumin excretion is shown. (G) Creatinine clearance normalized to 100g body weight based on 24 hour urine collection just prior to sacrifice at (day 62-63) is shown. N= 5-8 rats per group for panel B, D, F and G; N = 3 per group in panel E. Data are mean ±SEM. * indicates $P < 0.05$ vs Sham operated, # indicates $P < 0.05$ losartan vs. vehicle.

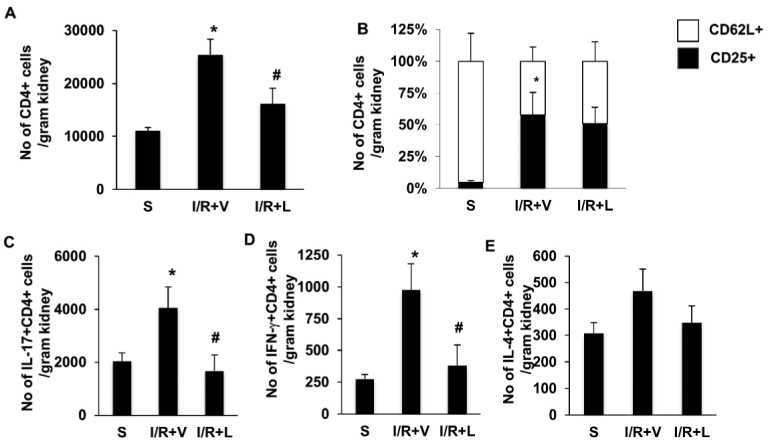

Figure 4. Effect of losartan on T cell infiltration and differentiation in post ischemic and sham rats

Lymphocytes were isolated from kidneys of sham-operated or injured animals with or without treatment with losartan and stained for markers of specific populations as in Figure 1. The number of CD4+ cells (A), proportion of CD25+ and CD62L cells, (B), IL-17+CD4+ cells (C), IFN-γ+CD4+ (D) and IL-4+CD4+ (E) are shown. N= 5-8 rats per group. Data are mean ±SEM. * indicates $P < 0.05$ vs Sham operated, # indicates $P < 0.05$ losartan vs. vehicle.

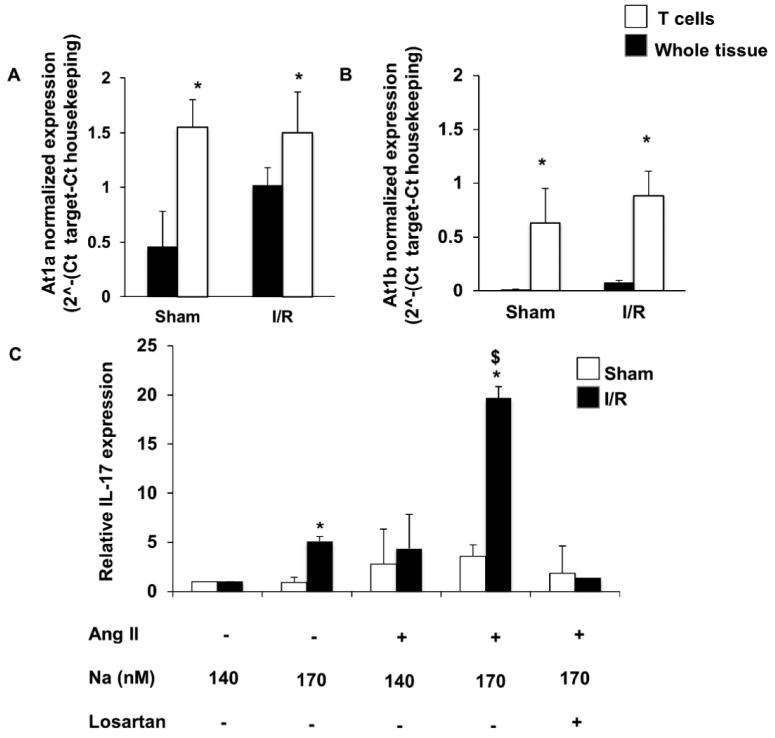

Figure 5. Effect Ang II and of elevated extracellular Na and on IL-17 secretion in isolated T cells

Panel A and B shows mRNA expression for AT1A and At1B receptor-measured in whole kidney tissue and isolated T cells in post ischemic rats fed high salt diet showing higher expression in T cells vs whole kidney tissue. N=4 per group, Data are mean ±SEM, * indicates $P < 0.05$ vs isolated cells vs whole tissue. Panel C derives from CD4+ T cells isolated from kidney of rats following sham surgery or ischemia reperfusion, placed *in vitro*, and stimulated as described in *Methods*. IL-17 mRNA was measured in response to overnight stimulation with either Ang II (10^{-7}M), or a change in the extracellular Na^+ concentration to (170mM), with or without losartan (1μM). Expression levels of IL-17 mRNA in response to treatments are shown. Data are mean ±SEM and are derived from N=3 independent cultures; * indicates $P < 0.05$ vs control (ie., 140 mM Na^+, no added Ang II), $ indicates $P < 0.05$ vs either Ang II at 140 mM Na or no Ang II with 170 mM Na.