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## **Calcium channel Orai1 promotes lymphocyte IL17 expression and progressive kidney injury**

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Conflicts of Interest:

DPB, PM and MS are authors on a patent application “Methods to treat renal disorders using calcium channel inhibitors”, US Provisional Application Serial No. 62/741,302, filed October 4, 2018.

MS is Co-founder and Chief Scientific Officer of Corvus Biomedical LLC.

Running Title: Orai-1 mediated Ca<sup>2+</sup> promotes AKI and CKD progression

**ABSTRACT**

We hypothesized that the store operated calcium entry (SOCE) channel, Orai1, participates in the activation of T-helper (Th17) cells and influences renal injury. In rats following renal ischemia/reperfusion (I/R), there was a rapid and sustained influx of Orai1+ CD4 T-cells and IL17 expression was restricted to Orai1-positive cells. When kidney CD4+ cells of post-AKI rats were stimulated with angiotensin II and elevated Na+ ( $10^{-7}$ M/170 mM) in vitro, there was an enhanced response in intracellular Ca<sup>2+</sup> and IL17 expression, which was blocked by SOCE inhibitors 2APB, YM58483/BTP2, or AnCoA4. In vivo, YM58343/BTP2 (1 mg · kg<sup>-1</sup>) attenuated IL17+ cell activation, inflammation and severity of AKI following either I/R or intramuscular glycerol injection. Rats treated with high-salt diet (5-9 weeks post I/R) manifested progressive disease indicated by enhanced inflammation, fibrosis and impaired renal function. These responses were significantly attenuated by YM58343/BTP2. In peripheral blood of critically ill patients, Orai1+ cells were significantly elevated by ~10-fold and Th17 cells were elevated by ~4 fold in AKI vs non-AKI patients. Further, in vitro stimulation of CD4+ cells from AKI patients increased IL17, which was blocked by SOCE inhibitors. These data suggest that Orai1 SOCE is a potential therapeutic target in AKI and CKD progression.

**INTRODUCTION:**

Acute kidney injury (AKI) results from events such as renal ischemia, nephrotoxicity and/or sepsis. AKI increases the risk of death in the intensive care unit (ICU), and mortality rates in this setting range between 15-60% (1-3). Survival from AKI is dependent on recovery of renal function following injury, the success of which has been suggested to be dependent on the efficiency of adaptive repair processes (4, 5). Progression of chronic kidney disease (CKD) and end-stage kidney disease is recognized as a possible outcome of AKI patients, and it has been suggested that incomplete or maladaptive repair may predispose progression of CKD following AKI (4, 5).

Immune cell activity may contribute to renal injury or may enhance renal recovery. In the setting of renal ischemia reperfusion (I/R) injury, renal CD4+ T-helper 1 or 17 cells are thought to exacerbate renal injury while T-regulatory cells have been implicated in renal repair (6, 7). Following recovery from I/R injury in rats, subsequent exposure to high-salt diet was shown to hasten the development of interstitial fibrosis, inflammation, proteinuria and hypertension. These parameters of CKD progression were significantly attenuated by immunosuppression with mycophenolate (8, 9), suggesting that lymphocyte activity also modulates the AKI-to-CKD transition.

Naïve CD4+ cells differentiate into effector T-helper cells in the ischemic milieu, where they are exposed to different antigens and pro-inflammatory cytokines. T-helper cells secrete various cytokines and are thought to orchestrate the adaptive immune response (6). Our group demonstrated that T-helper 17 (Th17) cells, which secrete the cytokine IL17, are the prominent lymphocyte population found in rat kidney following I/R injury (10). These cells have been implicated in a variety of autoimmune diseases such as asthma, psoriasis, inflammatory bowel disease and lupus erythematosus (11). There is a significant expansion of Th17 cells in kidney within the first 3 days of I/R injury in rats, while Th17 levels resolve to near sham-operated control values within 7 days as renal function recovers. However, subsequent exposure of rats to high salt diet (4%) strongly reactivates Th17 cell expression in post-ischemic kidney (10). This re-activation may contribute to CKD, since an IL17R antagonist attenuated renal interstitial fibrosis and neutrophil infiltration in post I/R rats exposed to high salt diet (12).

The basis for activation of Th17 cells in response to renal injury and exposure to

high-salt diet remains to be elucidated. Th17 cell differentiation is dependent on the activity of the transcription factor ROR $\gamma$ T and inhibitors of this factor can alleviate the pathological activation of Th17 cells (13). Activation of these cells by high salt diet has also been demonstrated in a mouse model of autoimmune encephalitis and associated with the activity of serum and glucocorticoid regulated kinase (SGK-1) and nuclear factor of activated T-cells 5 (NFAT5) (14). Elevation of extracellular Na<sup>+</sup> to 170 mM enhanced differentiation from naïve CD4<sup>+</sup> cells to Th17 cells *in vitro* in a process dependent SGK-1(15).

Using an *in vitro* stimulation assay, we demonstrated that CD4<sup>+</sup> T cells from post-ischemic kidney manifest enhanced expression of IL17 in response to Ang II and elevated extracellular Na<sup>+</sup> *in vitro*, while no response was observed from sham-operated control derived CD4 cells (12). The mechanisms mediating the enhanced IL17 activation in CD4 cells post AKI are not known. Previous studies have also demonstrated that Orai1, the pore forming subunit of Ca<sup>2+</sup> release-activated Ca<sup>2+</sup> channels (CRAC), is required for Th17 cell differentiation *in vitro*, due in part to NFAT activity (16, 17). Interestingly, Orai1 mutant mice, or inhibitors of Orai1 show impaired T-cell receptor (TCR) activation, reduced IL17 production and are resistant to autoimmune disorders (17). Therefore, we tested the hypothesis that renal I/R enhances lymphocyte Orai1 mediated Ca<sup>2+</sup> signaling that drives Th17 cell expression and, in turn, modulates AKI and AKI-to-CKD progression.

## RESULTS

Previous studies demonstrated that Th17 cells are rapidly induced following renal I/R and that IL17 contributes to AKI (10, 18). To investigate the potential that Orai1 participates in AKI, Orai1 expression was measured in Th17 cells from kidneys of rats 2 days following sham or I/R injury (*Study I*). Orai1 was detected in Th17 cells and the number of these cells was increased following I/R relative to sham (**Figure 1A**). When accounting for influx, the total number CD4<sup>+</sup>/Orai1<sup>+</sup> cells and the number of triple-positive CD4<sup>+</sup>/IL17<sup>+</sup>/Orai1<sup>+</sup> cells in kidney were markedly elevated by I/R injury (**Figure 1B & C**). In CD4 cells, Orai1 was associated with increased IL17 signal (**Figure 1D**) and IL17<sup>+</sup> cells were found almost exclusively in Orai1<sup>+</sup> cells in both sham and post I/R groups (**Figure 1E**). Orai1 expression was also observed in CD8<sup>+</sup>, B-cells, NK cells and macrophages but the percent of these populations was modest when compared with CD4 cells (**Supplemental Table 2**). Orai1 has 2 homologs referred to Orai2 and Orai3, which have been suggested to modulate lymphocyte responses (19, 20). However, neither Orai2 nor Orai3 were significantly affected by I/R and neither Orai2<sup>+</sup> nor Orai3<sup>+</sup> cells co-expressed IL-17 (**Supplemental Figure 2**).

Kidney Th17 levels return to sham-operated control values within ~7 days of I/R (10). Despite the reduction of Th17 cells, Orai1 expression was maintained in CD4<sup>+</sup> cells 7 days post I/R (**Figure 1F**). Post-AKI rat kidneys also demonstrate a greater percentage of CD4 cells expressing the IL17 transcription factor, ROR $\gamma$ T (**Supplemental Figure 3A**). When placed in culture, these AKI-primed CD4<sup>+</sup> cells (7 days post I/R), but not sham CD4<sup>+</sup> cells, increase IL17 mRNA expression following in vitro stimulation with Ang II and elevated Na<sup>+</sup> (10<sup>-7</sup>M/170 mM) (10) (**Supplemental Fig 3B**). This treatment also significantly increases the percentage of IL17-expressing cells from ~12% to ~49% as detected by FACS (**Figure 2A and B**). This response specifically requires elevated Na<sup>+</sup>, since increasing osmolality to a similar degree with either mannitol or choline chloride does not induce IL17 mRNA in the presence of Ang II (**Supplemental Fig 3B**). The IL17<sup>+</sup> cells induced following treatment co-express ROR $\gamma$ T suggesting activation of a predominately Th17 phenotype (**Supplementary Figure 3C**).

Kidney derived CD4<sup>+</sup> cells were examined further for markers of effector memory T cells (CD44<sup>+</sup>/CD62L<sup>-</sup>) 7 days following I/R injury. There was a ~4-fold increase in such cells from post I/R rats vs sham (1.85±0.01 % vs 7.65±1.23 %; p< 0.05). Stimulation with Ang II and elevated Na<sup>+</sup> did not affect the percentage CD44<sup>+</sup> effector memory T cells suggesting this population is not responsive to stimulation that promotes IL17 expression (**Supplemental Figure 3D**).

To evaluate a potential role for Orai1 in the IL17 response, AKI-primed CD4<sup>+</sup> cells were stimulated with Ang II and elevated Na<sup>+</sup> in the presence or absence of different SOCE inhibitors. Both 2-ABP and YM58483/BPT2 completely blocked the increase of IL17 mRNA as well as the increase in IL17<sup>+</sup> cells (**Figure 2B and C**). In addition, AnCoA4, an inhibitor considered to be highly specific for Orai1 due to its binding to stromal interaction molecule 1 (STIM1) and thereby inhibiting gating of the Orai1 channel (21), also completely blocked the induction of IL17 mRNA and protein.

To evaluate Orai1 activity in AKI-primed CD4<sup>+</sup> cells further, intracellular free Ca<sup>2+</sup> responses were evaluated with the fluorescent indicator fura-2. Representative tracings of sham-operated and AKI-primed CD4<sup>+</sup> cells are shown in (**Figure 2D**). When the superfusate was changed to a buffer containing Ang II and elevated Na<sup>+</sup>, a rapid and sustained increase in cytosolic Ca<sup>2+</sup> was observed in a significant percentage of AKI-primed lymphocytes when compared to lymphocytes derived from sham-operated controls (**Fig 2D and E**). The addition of either AnCoA4 or YM58343/BPT2 significantly attenuated the percentage of Ca<sup>2+</sup>-responding cells to levels similar to sham.

To evaluate if SOCE influences Th17 cells in AKI, rats were fed YM58483/BPT2 approximately 2 hours prior to 40 min of I/R (*Study II*). YM58483/BPT2 significantly attenuated the level of renal injury 24 hours after reperfusion as indicated by the level of plasma creatinine (**Figure 3A**) and mRNA expression of kidney injury marker-1 (Kim-1) (**Figure 3B**). YM58483/2-BPT also attenuated the infiltration of total CD4<sup>+</sup>T-cells, B-cells, and dendritic cells following I/R (**Figure 3C-F**). Total IL17 expressing cells were significantly reduced by approximately ~78% in YM58483/BPT2 treated rats relative to vehicle (**Figure 3G**) and this reduction of IL17<sup>+</sup> was primarily observed in the CD4 population (**Figure 3H**) while YM58483/BPT2 did not significantly effect CD8<sup>+</sup> cells or

CD8+IL17+ cells (**Figure 3 D and I**). Also, YM58483/BPT2 did not significantly influence either Th1 (IFN- $\gamma$ ; **Figure 3J**) or Th2 (IL-4+; data not shown) cells post-ischemia.

Orai1 is present in immune cells, but may also be present in other cell types such as vascular cells. We sought to determine if the effect of YM58483/BPT on renal injury was primarily due to its effects on Th17 activation. To address this, rats were subjected to bilateral renal I/R and treated with either YM58483/BPT2, the IL17Rc receptor antagonist or both. Consistent with our previous data, YM58483/BPT2 treatment alone significantly reduced plasma creatinine as compared to vehicle controls in post ischemic rats, however IL-17Rc+YM58483/BPT2 treatment did not have any additional effect (**Table 1**). Levels of kidney CD4+ and CD4+/IL17+ cells were significantly reduced vs vehicle-treated rats by a similar degree in both YM58483/BPT and IL17Rc treated rats (**Table 1**). When rats were treated with a combination of YM58483/BPT and IL17Rc, the reduction in renal injury was similar to that observed with either YM58483/BPT or IL17Rc alone (**Table 1**). The lack of an additional effect of IL17Rc suggests that the primary effect of YM58483/BPT is due to inhibition of Th17 cells in the early post-ischemic period.

To explore further whether SOCE affects Th17 differentiation and renal injury, YM58483/BPT2 was tested in a different model of AKI associated with rhabdomyolysis. Intramuscular injection of glycerol into water deprived rats resulted in severe renal injury (**Table 2**). In this model, renal CD8+ cells including CD8+/IL17+ cells appeared to be the predominant lymphocyte population, as opposed to the more prevalent CD4+ lymphocyte response following I/R. Nevertheless, YM58483/BPT2 significantly attenuated the rise in serum creatinine as well as the total number of IL17+ expressing cells (**Table 2**).

Exposure of rats to high-salt diet at 5 weeks following recovery from I/R re-stimulates renal Th17 cell activity that is thought to contribute to CKD progression (10). The effect of SOCE on Th17 activation by high salt diet following AKI was evaluated in rats subjected to unilateral renal I/R followed by contralateral nephrectomy and transition to high salt diet (*Study III*). Post AKI rats treated with vehicle during high salt diet treatment manifested a significant infiltration of kidney CD4+ and CD8+ cells and IL-17 expressing cells. YM58483/BPT2 treatment significantly attenuated the increase in CD4+ and CD8+ cells and the increased expression of IL17+ cells (**Figure 4A-D**). YM58483/BPT2 also significantly attenuated the increase in B cells and M $\phi$ /dendritic cells in post-AKI rats fed

high salt diet (**Figure 4E and F**). Creatinine clearance at the end of the study period (i.e., 9 weeks post I/R) was significantly reduced in vehicle-treated I/R rats relative to sham-operated controls but creatinine clearance was not reduced in YM58483/BPT2 treated rats relative to sham (**Figure 4G**). Post-ischemic rats treated with vehicle also showed significant alterations in other parameters related to CKD including urinary albumin excretion, the development of interstitial fibrosis, and the expression of Kim-1; these parameters were all significantly attenuated in YM58483/BPT2-treated rats (**Figure 4H and 4I and Suppl Fig 4**).

We previously demonstrated increased circulating Th17 cells in rat blood following I/R, suggesting that blood may be used as source of activated lymphocytes in the setting of AKI (22). To investigate the potential that SOCE influences Th17 differentiation in human AKI, peripheral blood samples were obtained from critically ill patients with and without AKI. Samples were collected within 24-48 hours of AKI diagnosis for AKI cases or within 24-48 hours of ICU admission for frequency-matched (age, gender, baseline eGFR) controls without AKI (**Table 3**). In isolated blood mononuclear cells, the percentages of total IL17+ cells and CD4+/IL17+ cells were significantly higher in AKI patients vs non-AKI patients (**Figure 5A-C**). Moreover, the percentage of Orai1 positive cells was also prominently increased from ~3% in non-AKI patients to ~30% in patients with AKI. Similar to studies in rat kidney, Th17 cells were predominantly found within Orai1 expressing cells vs Orai1-negative cells (**Figure 5D-F**).

We next examined whether AKI enhanced IL-17 responses in circulating human CD4 cells and if these responses depend on SOCE activity. Human CD4+ cells responded *in vitro* to elevated extracellular sodium by increasing IL-17 expression in samples from AKI patients, but not in those of patients without AKI (**Figure 5G**). In contrast to rat kidney CD4+ cells, human blood CD4+ cells responded to elevated extracellular Na+ alone and did not require the addition of Ang II. Importantly, the IL17 response appeared dependent on SOCE activity, since both YM58483/BPT2 and AnCoA4 inhibited this response (**Figure 5G**).

## Discussion

In this study, we propose a novel regulatory pathway related to the activation of lymphocytes in the setting of acute and chronic kidney injury based on the role of intracellular calcium signaling and the differentiation of Th17 cells. Our studies demonstrate that the store-operated  $\text{Ca}^{2+}$  channel Orai1 is prominently induced in renal T-cells in the setting of kidney injury. Moreover, blockade of this channel attenuated Th17 cell induction and renal damage in response to ischemia/reperfusion injury as well as subsequent exposure to high salt diet. Thus, Orai1 may represent a therapeutic target to attenuate AKI or immune mediated renal fibrosis and hypertension, which may occur secondary to AKI.

Th17 cells were originally described as a distinct T-helper subset which secretes the cytokine IL-17 and is a major factor in autoimmune disorders. Th17 cells play an important role in host defense. However, in models of asthma, inflammatory bowel disease, psoriasis, or autoimmune encephalitis, Th17 cells aggravate inflammation by recruitment of other immune cells (such as neutrophils), which express the IL17RA receptor (23). Th17 cells have also received significant attention in the setting of renal inflammatory disorders including ANCA associated vasculitis (24), crescentic glomerular nephritis (25) and nephrotic syndrome (26, 27). Following renal transplant, there is an increased prevalence of Th17 cells in patients with chronic allograft nephropathy (28).

Th17 cells have recently been examined in the setting of AKI. Studies using *IL17-null* mice, suggest that Th17 cells contribute to the severity of renal injury in response to I/R (29) or cisplatin (18). Work from our group demonstrated a biphasic Th17 response in rats, with an early transient phase of expression peaking between 1-3 days following injury and the second peak induced when rats are provided high-salt diet. Th17 cells were the predominant lymphocyte population activated by high salt diet while no significant effect was observed on Th1 or Th2 cells (12). The exposure to high salt diet exacerbates inflammation, fibrosis and hypertension and can be attenuated by mycophenolate or an IL17 antagonist (8, 12). Therefore, the mechanisms mediating IL17 expression in response to I/R and high salt intake are of interest. An *in vitro* model using CD4<sup>+</sup> cells from kidneys 7 days following I/R revealed that stimulation by Ang II and elevated extracellular sodium increased IL17 expression, while having no effect on Th1 or Th2

responses (12). This priming of CD4<sup>+</sup> cells by AKI provided an opportunity to investigate altered lymphocyte signaling leading to Th17 differentiation in response to renal injury and was the basis of the current study.

Kleinewietfeld et al. investigated the potential mechanism by which Na<sup>+</sup> may directly influence Th17 cell differentiation. These authors demonstrated that naïve T-cells, when cultured for 4 days with TGF- $\beta$  and IL6, differentiate into Th17 cells and this response was potentiated elevating the concentration of Na<sup>+</sup> in the culture media. In addition, the response was abrogated by inhibition of p38/MAPK, NFAT5 and SGK-1. It was suggested that the increase in Na<sup>+</sup> concentration by up to an additional 40 mM might be observed in skin under high salt diet conditions and potentially influence T-cell function. Indeed, Th17 induction and severity of autoimmune encephalopathy was enhanced when mice were placed on a high salt diet (14).

TCR stimulation invokes an increase in intracellular Ca<sup>2+</sup> via Ca<sup>2+</sup>-release activated Ca<sup>2+</sup> channels (CRAC). The result of this activity is thought to be calcineurin mediated dephosphorylation of NFAT, which translocates to the nucleus and activates transcriptional programs. Genome wide RNAi screens helped to identify Orai1 as the pore forming subunit of CRAC channels (16, 30). Activation of Orai1 is mediated by the activity of STIM1, an endoplasmic reticulum (ER) membrane spanning protein which senses Ca<sup>2+</sup> depletion from the ER secondary to TCR stimulation. Interaction between STIM1 and Orai1 increases CRAC activity and results in sustained increases in intracellular Ca<sup>2+</sup> (31). Mutations in either *ORAI1* or *STIM1* result in a severe combined immunodeficiency (SCID) phenotype (32). For Th17 cell differentiation, TCR stimulation in combination with various other cytokines represents an important driver of differentiation, which is considered more inflammatory than Th1 or Th2 phenotypes. Interestingly, chemical library screening recently identified putative Orai1 inhibitors, which showed greater selectivity in abrogating Th17 differentiation vs Th1 or Th2 differentiation. Moreover, Orai1 inhibition reduced the nuclear accumulation of NFAT and ROR $\gamma$ T, critical transcriptional regulators of Th17 differentiation (16).

Data from the current study provide compelling evidence for an essential role of Orai1 in the differentiation of Th17 cells following renal I/R. IL17<sup>+</sup> expressing CD4<sup>+</sup> cells are rapidly expanded following renal injury and, using FACS analysis, we show that Orai1

expression is also prominent in kidney CD4+ cells following renal injury. Measurements from circulating blood of critically ill patients with AKI also demonstrated a profound enhancement of Orai1+ IL17+ cells, indicating that this pathway is activated in human AKI.

Importantly, IL17 expression was almost exclusive to cells expressing Orai1, and was essentially absent in Orai1-negative cells. Published reports suggest that Orai1 mutations result in impaired production of Th17 cells. The data from the current study indicate that Orai1 is persistently expressed following the resolution of AKI (7 days post I/R). Given that the re-expression of IL17 following *in vitro* stimulation is inhibited by SOCE antagonist, the results suggest that Orai1 mediated SOCE channel is required for Th17 differentiation following I/R.

The potential ability to target IL17 has already shown promise in the setting of autoimmune disease and several therapeutic agents are in use to target this pathway in diseases such as psoriasis (33). IL17 could also represent a target for both acute and chronic kidney disease since both have been shown to be ameliorated by IL17 blockade or *Il17* gene knockout strategies (9, 18). Similarly, we confirmed that a SOCE antagonist showed a significant degree of protection using a standard model of AKI in rats induced by bilateral renal I/R and also extended this observation to a model of AKI secondary to rhabdomyolysis. The protection was associated with a clear reduction in the genesis of Th17 cells in response to injury. However, since Orai1 may be expressed in endothelial cells or smooth muscle cells (34), we cannot exclude the possibility that the effects observed may be independent of IL17 production. Nevertheless, in the current study, IL17Rc blockade provided no additional protection over YM58483/BPT2 alone suggesting that the primary activity of Orai1 in the early post I/R period is to promote Th17 activation.

The transition from acute to chronic kidney disease has been the subject of significant research and maladaptive repair responses predispose CKD progression (4). Since immune suppression strongly attenuates the AKI to CKD transition, persistent inflammation in the kidney in response to AKI represents a potential maladaptive response. It is therefore noteworthy that Orai1 expression remains persistently elevated in CD4 cells despite the recovery of kidney function and the decline in IL17 expression. The degree and duration of sustained Orai1 expression following recovery from AKI remains unclear, but it is worth noting that reactivation of Th17 cells by high salt diet was

attenuated by SOCE inhibition between 35-63 days post injury. Whether Orai1 persists in activated T cells, memory T cells, or other leukocyte populations which secrete IL17 remains to be determined. Our studies demonstrated an increase in effector memory T cells 7 days following I/R. However, this population was not affected by *in vitro* stimulation so it is not yet clear whether this population contributes directly to the IL17 response post injury. Nevertheless, we suggest that sustained Orai1 expression may represent the basis for susceptibility to re-activation of Th17 cells and therefore represents an important link predisposing to salt-sensitive CKD progression following AKI.

In addition to providing a potential link between AKI-to-CKD, there is also clear evidence that CKD predisposes to AKI (35). It could be suggested that sustained expression of Orai1 in CKD could enhance sensitivity to AKI, by promoting a greater inflammatory response to a given insult. It is also reasonable to suggest that increased Orai1 expression enhances the IL17 response to other inputs. For example, in the current study, IL17 expression was elevated in response to Ang II and elevated Na<sup>+</sup> *in vitro* and was dependent on SOCE activity. Recent studies have shown that Ang II-dependent hypertension is, in part, dependent on IL17 activity (36, 37); whether Orai1 modulates Ang II dependent Th17 responses in these models remains to be determined.

Taken together, the results from the current study suggest that Orai1 could be considered a novel pathway to target inflammatory renal disease associated with the Th17 phenotype. Novel inhibitors targeting this pathway are currently in development (38) and could represent therapies for inflammatory diseases associated with Th17 cells including autoimmune disease as well as AKI, CKD and salt-sensitive hypertension.

## METHODS

### *Animal Study protocols*

All studies used male Sprague-Dawley rats (250–300g) that were purchased from Envigo (Indianapolis, IN). Rats were anesthetized with a cocktail of ketamine (100 mg · kg<sup>-1</sup>, KetoVed, Vedco Inc, St. Joseph, MO) and xylazine (50 mg · kg<sup>-1</sup>, AnaSed, Lloyd Inc, Shenandoah IA). Rats were placed on a heated surgical table to maintain body temperature. A midline incision and either unilateral (left) or bilateral renal ischemia (as indicated in *Results*) was induced by applying micro-aneurism clamps on the renal pedicles for a period of 40 min. Re-establishment of perfusion was verified by visual examination following removal of the clamps. Rats were provided post-operative analgesia using buprenorphine-SR (1 mg · kg<sup>-1</sup>).

Rats were allowed to recover for various periods of time as described in the *Results*. In *Study I*, lymphocytes were studied *in vitro* after recovery for either 2 or 7 days following I/R or sham surgery for evaluation of function *in vitro* or for FACS analysis. In *Study II*, the effect of SOCE on kidney injury was studied with the inhibitor YM58483/BPT2 (*N*-[4-[3,5-Bis(trifluoromethyl)-1*H*-pyrazol-1-yl]phenyl]-4-methyl-1,2,3-thiadiazole-5-carboxamide; Tocris/Bio-Techne Minneapolis, MN). Rats were pretreated p.o. 2-3 hours prior to surgery with YM58483.BPT2 (1 mg · kg<sup>-1</sup>) in sugar-free chocolate pudding following dilution in 100% EtOH, at a dose previously shown to affect T cell activation in rats (39). In some experiments, rats were also treated with the IL17Rc (150 ng/day i.p.; R&D Systems). In addition, one experiment utilized a model of rhabdomyolysis, induced by injection of 50% glycerol (10 ml/kg) into the hindlimb muscle of rats following 16 hours of water restriction as described previously (40).

*Study III* was designed to investigate SOCE on progression of CKD induced by high-salt diet following recovery from I/R injury using a model of AKI-to-CKD described previously (41). Rats acclimated to a standard diet (AIN 76A, Dyets, Bethlehem, PA) containing 0.4% NaCl were subjected to left unilateral I/R or sham surgery and allowed to recover for ~5 weeks. Rats were then subjected to right unilateral nephrectomy and subsequently exposed to elevated dietary Na<sup>+</sup> (AIN76A plus 4% NaCl) for an additional 4 weeks (**Supplemental Fig 4**). Sham-control rats were not subjected to renal pedicle

clamping, but were subjected to unilateral nephrectomy. Simultaneous with exposure to high salt diet, rats were randomly assigned to vehicle or YM58483 treatment ( $1 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ ; p.o.). At the end of all studies, rats were deeply anesthetized with 50-100 mg/kg pentobarbital (Fatal Plus, Vortech, Dearborn, MI) and kidneys harvested for analysis.

### *Human studies*

We designed a prospective case-control study to examine peripheral blood from AKI patients (n=9) and matched-controls without AKI (n=8) admitted to the ICU at the University of Kentucky Hospital. AKI was defined by KDIGO criteria (42), using both serum creatinine (SCr) and urine output data. Only patients with AKI stage  $\geq 2$  were included in the study as cases. Controls were frequency-matched by age (10-year intervals), gender and 2-category baseline estimated glomerular filtration rate (eGFR, calculated using CKD EPI equation,  $\geq 90$  and 60-89 ml/min/1.73 m<sup>2</sup>). Baseline SCr was defined as the most recent SCr within the 6-month period before ICU admission. Inclusion criteria included: adults  $\geq 18$  years of age, admission to the ICU, and baseline eGFR  $\geq 60$  ml/min/1.73 m<sup>2</sup>. Exclusion criteria consisted of prior kidney or any other solid organ transplant, end-stage kidney disease, evidence of AKI before ICU admission, or the presence of uroepithelial tumors.

Single-timepoint peripheral whole blood samples were obtained 24-48 hours after AKI diagnosis (cases) or ICU admission (controls). Standardized techniques for blood collection, transport and storage were employed.

### *Measurements of renal function*

To measure creatinine, tail blood was collected in heparin containing tubes and centrifuged to collect plasma. Plasma creatinine was measured using a Pointe Scientific Analyzer and Creatinine Assay reagents using methods outlined by the manufacturer (Pointe Scientific, Canton MI). Urine was collected for 24 hours by placing rats in metabolic cages and urine volume was determined gravimetrically. Urine creatinine was measured using a colorimetric assay adopted for microplate readers, as previously described (43). Creatinine clearance was measured using  $U_{Cr} \cdot V / P_{Cr}$ .

### *Renal histology, immunohistochemistry and RNA expression*

At the time of tissue harvest, kidneys were bisected and one half was fixed by immersion in 10% formalin, embedded in paraffin and 5  $\mu\text{m}$  sections stained with picosirus red to assess fibrosis. For quantitative analysis, five random images of renal outer medulla were obtained using Leica DMLB (Scientific Instruments, Columbus, OH) microscope with a 20x objective. The percent area of picosirus red stain was scored in a blinded fashion using Image J (NIH) using procedures described previously (10).

Total RNA was obtained from kidney using Trizol and the Zymogen RNA extraction kit and cDNA was prepared using MMLV enzyme (Invitrogen, Carlsburg, CA). Quantitative real time PCR (qPCR) using gene specific primers was performed using ABI 7500 (Applied Biosystems, Foster City, CA). mRNA values were calculated using  $2^{-\Delta\Delta\text{Ct}}$ . Specific primers sequences for IL-6 (catalog # Rn01410330\_m1), Kim-1 (# Rn00597703\_m1) and IL17 (Rn01757168\_m1) were purchased from ThermoFisher (Waltham, MA).

### *Rat CD4+ isolation and FACS analysis.*

Freshly harvested kidneys were minced and digested in liberase ( $2 \mu\text{g} \cdot \text{ml}^{-1}$ ; Roche, Indianapolis IN) for 15 min at  $37^\circ\text{C}$  using Gentle MACs (Miltenyli, San Diego, CA). The digested tissue was filtered through a 100- $\mu\text{m}$  mesh and washed with RPMI containing 10% fetal bovine serum (Invitrogen). Mononuclear cells were isolated using Percoll (Sigma) density gradient centrifugation.

All antibodies, their sources and concentrations used are listed in **Supplemental Table 1**. To evaluate protein expression of IL17 or Orai1, Orai2 or Orai3, cells were restimulated with PMA and ionomycin for 6 hours in the presence of monensin (GolgiStop,  $1 \mu\text{g} \cdot \text{ml}$ , BD Biosciences) permeabilized with saponin (10%) and stained with relevant antibody. Cells were scanned using flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA) and scans were analyzed using Flowjo software (Tree Star, Ashland, OR). Lymphocyte gating strategy was described previously (10) and a representative example of the gating strategy used in these studies is shown in **Supplemental Figure 1**.

*Functional T cell analysis (rat):*

CD4<sup>+</sup>T cells were isolated using the MACS Pan-T cell microbead separation kit (Miltenyl, Glabach, Germany). T cells were stimulated with plate bound anti-CD3 (precoated with 2 $\mu$ g/mL) and soluble anti-CD28 (1 $\mu$ g/mL). Cells (2.5 X 10<sup>5</sup> in 0.25 ml) were incubated 12-14 hours at 37°C in RPMI medium supplemented with 10% FBS (Invitrogen) in a 48-well plate. Cells were challenged with Ang II (Sigma, 10<sup>-7</sup>M) and raising the extracellular Na<sup>+</sup> from 140 mM to 170mM, using a 1 M NaCl solution. Calcium channel inhibitors 2ABP (10  $\mu$ M, Sigma) AnCoA4 (10  $\mu$ M, Millipore, Burlington, MA) and YM58483/BTP2 (10  $\mu$ M) were included to evaluate effects on IL-17.

To assess Ca<sup>2+</sup> responses, fura2 imaging was performed similar to methods previously described (44). Briefly, isolated CD4<sup>+</sup> T cells were loaded with fura-2AM (2.5  $\mu$ M, Sigma, St. Louis, MO) for 45 min, washed, placed on poly-lysine (Sigma) coated coverslips and placed in a superfusion chamber with physiological salt solution (PSS) containing 2 mM Ca<sup>2+</sup>. The chamber was mounted on an inverted epifluorescence microscope and signal measured with alternating excitation at 340 and 380 nm and emission at 510 nm using the InCa<sup>2+</sup>-imaging system (Intracellular Imaging Systems, Cincinnati, OH). Data were acquired at 1.5 Hz and representative tracings were smoothed to the 10 nearest neighbor points using GraphPad Prism. For analysis, an increase in signal intensity of > 1 standard deviation from baseline was considered a positive response. Frequency of responding cells was determined from an average 53  $\pm$  19 cells for each animal. Cells that did not respond to the Ca<sup>2+</sup> ionophore ionomycin (1  $\mu$ M) at the conclusion of the study were excluded from analysis.

*Human CD4<sup>+</sup> isolation and FACS analysis:*

Fresh blood cells from patients were washed with PBS twice and stimulated with PMA, Iono and monensin for 4-6 hours. Cells were stained for antibodies against IL-17, CD4 and Orai-1 (**Supplemental table 2**). The samples were initially blinded and diagnosis (AKI or non AKI) revealed after measurements of all samples were completed.

For in vitro stimulation of human blood cells, primary T cells were isolated from fresh blood using Straight whole blood CD4 kit from Miltenyi Biotech (Miltenyi, San Diego, CA) according to manufacturer's protocol. CD4+T cells were plated at a density of  $1 \times 10^6$  cells/mL in RPMI medium supplemented with FBS. Cells were stimulated with human anti-CD3/CD28 dynabeads (Gibco; Catalogue no 11161D) along with labeled treatment overnight (~12 hours). T cells were harvested and incubated with monensin for 6 hours, prior to staining for IL-17 (**Supplemental table 2**).

### *Statistical analysis*

All data are expressed as means  $\pm$  SE or SD or median [IQ1-IQ3]. For experimental data, differences in means were established by 1-tailed Student's *t*-test or one-way ANOVA with Tukey's multiple comparison test as indicated in the figure legends. For clinical data, comparisons were done using Fisher's exact test for categorical variables and Mann-Whitney U test for continuous variables. Analysis was done with the aid of Graph Pad Prism software (La Jolla, CA) or SAS 9.4 (SAS institute, Cary, NC).  $P < 0.05$  was considered significant.

### Study Approval

Rats were maintained in accordance with the policies of the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. All studies were approved by Institutional Animal Care and Use Committees at Indiana University School of Medicine, Indianapolis, IN. For human studies, the protocol was approved by the Institutional Review Board of the University of Kentucky, Lexington, KY. Informed consent was obtained for all study participants.

**AUTHOR CONTRIBUTIONS**

PM and DB were responsible for the design and analysis of all rat studies described and JAN participated in the design of studies involving human samples, the screening, selection and matching of patients and obtaining samples used in the study. MS participated in the experimental design, training and interpretation of Ca<sup>2+</sup> imaging studies and interpretation of studies using Ca<sup>2+</sup> inhibitors. PM conducted all experimental procedures and collected all data. DB assisted with animal surgeries. DB and PM were responsible for writing of the manuscript and MS and JAN reviewed and edited the manuscript.

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## Figure Legends

**Figure 1: Co-expression of Orai1 and IL17 in CD4+ T cells following renal I/R** A) Left; Contour plot of renal lymphocytes stained with antibodies for CD4 and IL17 to identify Th17 cells. Orai1 staining in gated Th17 cells of sham is shown in middle panel and 2 days following I/R in the right panel. B) The number of Orai1+/CD4+ cells in sham and post-I/R rat kidneys is shown. C) The number of Orai1+/CD4+/IL17+ cells in sham and post-I/R rat kidneys is shown. D) Representative histogram of Orai1+ cells in kidney CD4 fraction (left) 2 days following I/R and the distribution of IL17 expression as a function of Orai1 is shown on right. E) The percentage IL17+ cells as a function of Orai-1 expression in CD4+ cells following sham surgery or 2 days following I/R is shown. F) Sustained expression of Orai1 in CD4+ cells following 7 days of recovery from I/R surgery is shown as representative histogram (left) and total number of Orai1+/CD4 cells is shown in the right panel. In B, C, E and F, data are mean  $\pm$  SE of 4-5 rats per group; \* indicates  $P < 0.05$  sham vs post-AKI by Student's t-test. In panel E, \* indicates  $P < 0.05$  in sham vs. I/R, † indicates  $P < 0.05$  in Orai1- vs Orai1+ cells, by one-way ANOVA and Tukey's post-hoc test.

**Figure 2: Orai1 activity contributes to IL17 expression in CD4+ lymphocytes primed by renal ischemia/reperfusion injury.** A) Illustrates representative FACS showing increased IL-17 expression in CD4+ cells from 7 day post-AKI rats following stimulation in vitro with 170 mM Na<sup>+</sup> and Ang II vs. control media; B) percent IL17+ cells in CD4+ cells isolated 7 days following sham or AKI and stimulated in vitro; C) IL17 mRNA, expressed as  $2^{-\Delta\Delta CT}$  of kidney derived CD4+ cells isolated 7 days following I/R surgery and stimulated in vitro. In panel B and C, control refers to AKI-primed CD4+ cells stimulated with 170 mM Na<sup>+</sup> and Ang II ( $10^{-7}$ M), and SOCE inhibitors included as labeled. D) Fura-2 fluorescence imaging of intracellular Ca<sup>2+</sup> in CD4+ lymphocytes in response to increased Na<sup>+</sup> (170 mM) plus Ang II ( $10^{-7}$ M), as indicated in the timeline and expressed as the ratio of fluorescence using 340/380 nm excitation. Shown are representative tracings of CD4+ cells from kidney following sham surgery (black) or I/R injury (red), or from I/R injury with co-incubation with AnCoA4 (blue). The inset illustrates representative visual field of multiple fura-2 loaded cells. E) Percent of cells manifesting an increase Ca<sup>2+</sup> response relative to baseline following in vitro stimulation with increased Na/Ang II. Data are mean  $\pm$  SE from 4-5 rats per group per assay; \* indicates  $P < 0.05$  vs unstimulated cells (i.e., no Ang II and normal Na, data not shown, see Supplemental Figure 3); † indicates  $P < 0.05$  inhibitors vs stimulated post-AKI cells by one-ANOVA and Tukey's post-hoc test.

**Figure 3: YM58483 pretreatment attenuates renal ischemia reperfusion injury. A)** Assessment of renal function by plasma creatinine 24 hours following I/R in Sprague-Dawley rats pretreated with YM-58483 or vehicle. Values for sham rats were  $0.4 \pm 0.1$  mg/dl are not shown on graph. B) Kim-1 mRNA levels from total kidney RNA 24 hours following sham or I/R are shown, (N.D. = not detectable). Total kidney derived CD4+ and CD8+ cells are shown in C and D, respectively; total B cells (RT1B+) and dendritic/macrophage cells (DC/M $\phi$ ; CD11b/c+) are shown in E and F, respectively. The total number IL17+, CD4+/IL17+ and CD8+/IL17+ cells are shown in G, H, I respectively. CD4+/IFN $\gamma$ + cells are shown in J. Data are mean  $\pm$  S.E. (N=11rats/group in panel A and 7-8 rats in B-J). \*P<0.05 I/R vehicle vs sham; † P<0.05 vehicle vs YM58483 by one way ANOVA and Tukey's post-hoc test.

**Figure 4. YM58483 attenuates inflammation and progressive renal damage following I/R and exposure to high salt diet.** Shown are levels of renal CD4+ (A), CD8+ T cells (B), IL-17+ expressing cells (C), and CD4+/IL17+ cells (D). Levels of B cells (E) and dendritic cells/M $\phi$  (F) are shown. Creatinine clearance from 24 hour urine collections at day 62-63 is shown in panel G and urinary albumin/creatinine ratio is shown in panel H. Panel I illustrates representative picosirius red stained sections through renal outer medulla from sham, I/R vehicle or I/R + YM 58483-treated rats. Quantification of stained area is illustrated in J. Data are mean  $\pm$  S.E. (N=5-6 rats/group). \*P<0.05 I/R vehicle vs sham; † P<0.05 vehicle vs YM58483 by one way ANOVA and Tukey's post-hoc test.

**Figure 5. Elevated Th17 and Orai1 expression and effect of SOCE on IL17 responses in CD4+ blood lymphocytes in critical ill patients with vs without AKI.** A) Representative FACS analysis of total peripheral blood cells in non-AKI (left, n=8) vs AKI patients (right, n=9) demonstrating the increase in CD4+IL17+ expressing cells. Quantification of the percentages of total IL17+ cells (B) and CD4+/IL17+ cells (C) in AKI vs non-AKI patients are shown. Representative dot plot showing an increase in Orai-1+ cells in AKI patients as compared to non-AKI patients is shown in (D) and the percentage of Orai-1+ cells in AKI vs non-AKI patients is shown in (E). (F) The %CD4+/IL17+ gated on Orai1+ and Orai1- fraction (arrows) is shown. In panel G, IL17 response to stimulation with elevated sodium (170 mM) and Ang II is shown for CD4+ cells isolated from AKI (n=5) or non-AKI (n=5) patients. Data are expressed as mean  $\pm$  S.E. In panel B, C and D \* indicates P<0.05 in AKI vs non-AKI patients by Student's t-test. In panel F, \* indicates p<0.05 in Orai1+ vs Orai1- cells. In G, \* indicates p< 0.05 vs unstimulated (i.e., 140 mM and no Ang II), † p<0.05 inhibitors vs stimulated using one-way ANOVA and Tukey's post-hoc test.

**Table 1. Effect of SOCE inhibitor YM58343 and IL17Rc on renal injury and renal lymphocyte content 24 hours following ischemia reperfusion.**

	Sham (n=3)	I/R+Vehicle (n=6)	I/R+Ym-58343 (n=5)	I/R+IL-17Rc (n=8)	I/R+YM+IL-Rc (n=8)
Serum Creatinine (mg/dl)	0.7±0.1	5.1±0.8*	3.7±0.6 †	3.96±1.2	3.9±1.0
CD4+ (cells/gram)	19590±2812	118964±11193*	26156±4365 †	19229±3175†	18636±3140†
CD8+ cells (cells/gram)	2446±295	31237±5362*	8410±1386†	4356±559†	4929±974†
Total IL-17+ (cells/gram)	4117±671	17887±14133*	9334±4293†	8447±1753†	8718±1630†
CD4+/IL-17+ (cells/gram)	1937±540	8111±3903*	2020±731†	1567±294†	1694±262†
CD8+IL-17+ (cells/gram)	30±1.1	1647±770*	127±23†	251±118†	281±173†

Data are mean ± S.E; \* indicates p<0.05 I/R vehicle vs sham. † indicates p<0.05 vs vehicle by one-way ANOVA and Tukey's multiple comparison test.

**Table 2. Effect of SOCE inhibitor YM58343 on glycerol-induced kidney injury and lymphocyte content 24 hours following injection.**

	Glycerol+ Vehicle (n=5)	Glycerol+YM58343 (n=5)
Serum Creatinine (mg/dl)	4.2 ± 0.3	3.3 ± 0.4*
CD4+ cells per g	234294 ± 24717	177606 ± 282
CD8+ cells per g	552129 ± 139124	342433 ± 76011
Total IL-17+ cells per g	112343 ± 19217	60109 ± 21033*
CD4+/IL-17+ cells per g	34972 ± 3108	20622 ± 4191*
CD8+/IL-17+ cells per g	57667 ± 1820	19711 ± 6499*

Data are mean ± S.E; \* indicate  $p < 0.05$  by Student's t-test

**Table 3. Characteristics of Patients according to AKI status**

	Total	AKI	Controls	P-value
Number of patients	17	9	8	
<b>Demographics</b>				
Age, years, mean $\pm$ SD	63.9 $\pm$ 10.9	63.8 $\pm$ 12.0	64.1 $\pm$ 10.4	0.063
Male, n (%)	11	7 (77.8)	4 (50.0)	0.232
White race, n (%)	15	9 (100.0)	6 (75.0)	0.110
BMI, kg/m <sup>2</sup> , median [IQ1-IQ3]	26.0 [23.1-34.4]	33.9 [24.9-35.9]	25.9 [24.5-28.9]	0.481
<b>Baseline kidney function</b>				
eGFR, ml/min/1.73m <sup>2</sup> , median [IQ1-IQ3]	89.0 [74.2-106.1]	83.2 [75.4-89.0]	100.7 [91.1-105.1]	0.481
SCr, mg/dl, median [IQ1-IQ3]	0.7 [0.6-0.9]	0.9 [0.7-1.0]	0.7 [0.6-0.8]	0.093
<b>Comorbidity</b>				
Charlson Comorbidity score, median [IQ1-IQ3]	3.0 [2.0-4.5]	3.0 [2.0-5.0]	3.0 [2.8-3.3]	0.888
Diabetes, n (%)	8	6 (66.7)	2 (25.0)	0.086
Hypertension, n (%)	14	7 (77.8)	7 (87.5)	0.600
Congestive heart failure, n (%)	2	1 (11.1)	1 (12.5)	0.929
COPD, n (%)	6	2 (22.2)	4 (50.0)	0.232
Liver disease, n (%)	2	1 (11.1)	1 (12.5)	0.929
Anemia, n (%)	7	3 (33.3)	4 (50.0)	0.486
Cancer, n (%)	6	3 (33.3)	3 (37.5)	0.858
<b>AKI characteristics</b>				
Peak SCr, mg/dl, median [IQ1-IQ3]		2.9 [2.5-3.7]	--	
KDIGO stage of AKI				
Stage 2, n (%)		3 (33.3)	--	
Stage 3		6 (66.7)	--	
<b>Critical illness parameters</b>				
CFB 72 hours, liters, median [IQ1-IQ3]	3.0 [0.8-4.3]	3.7 [3.0-4.8]	2.1 [0.8-3.0]	0.139
CFB ICU stay, liters, median [IQ1-IQ3]	8.3 [2.7-25.6]	14.7 [6.4-35.4]	3.6 [3.1-6.3]	0.268
Pressor or inotrope, n (%)	8	8 (88.9)	0 (0.0)	<0.001
Mechanical ventilation, n (%)	12	8 (88.9)	4 (50.0)	0.079
APACHE II score, median [IQ1-IQ3]	20.0 [14.5-26.0]	26.0 [21.0-32.0]	14.5 [12.0-17.3]	<0.001
SOFA score, median [IQ1-IQ3]	7.0 [3.5-10.0]	10.0 [8.0-11.0]	3.5 [2.8-6.3]	0.002

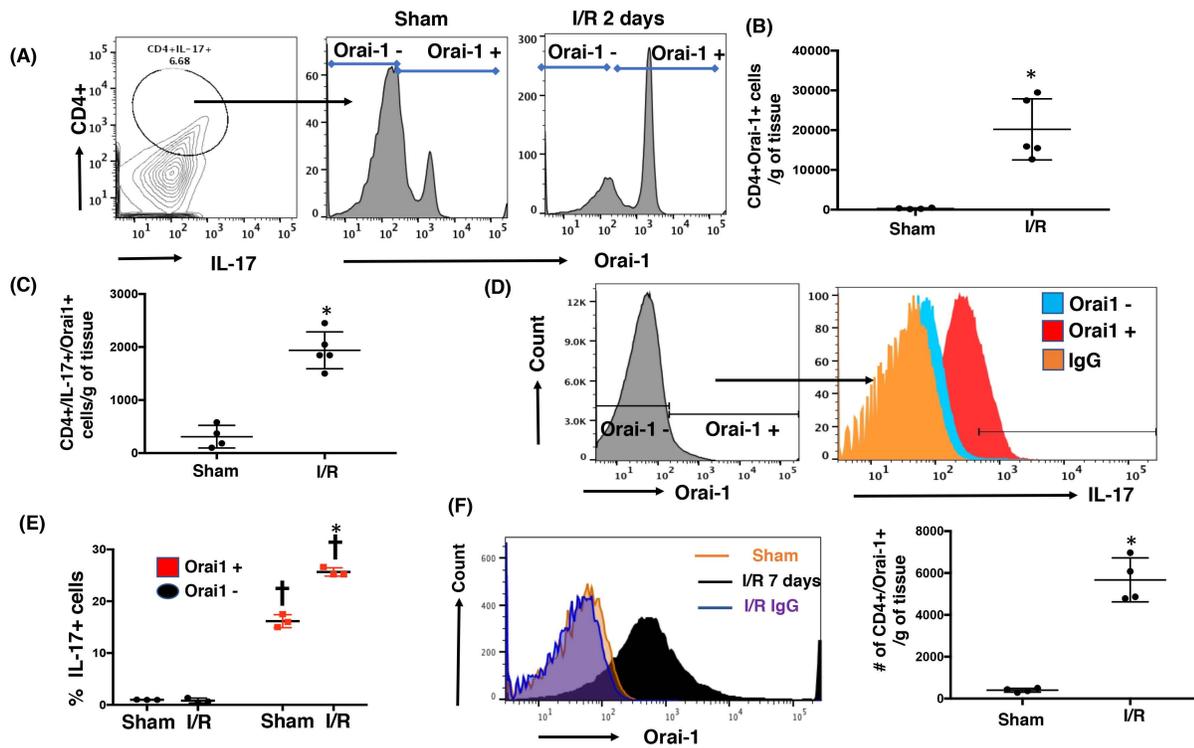
Comparisons were done using Fisher's exact test for categorical variables and Mann-Whitney U test for continuous variables.

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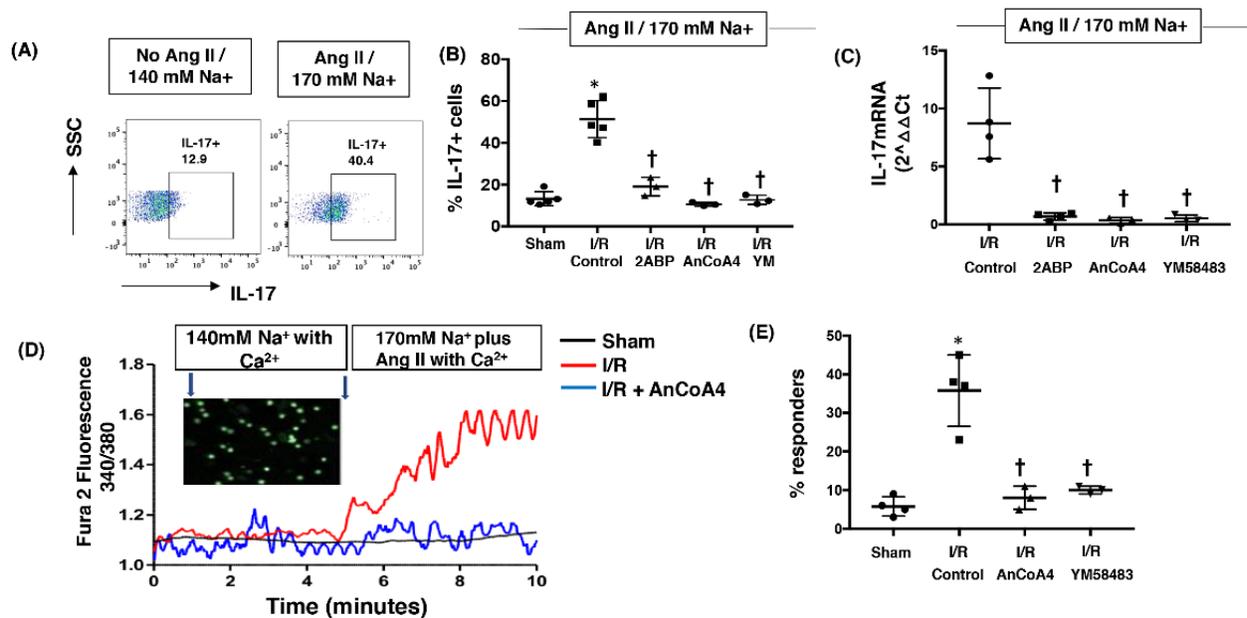
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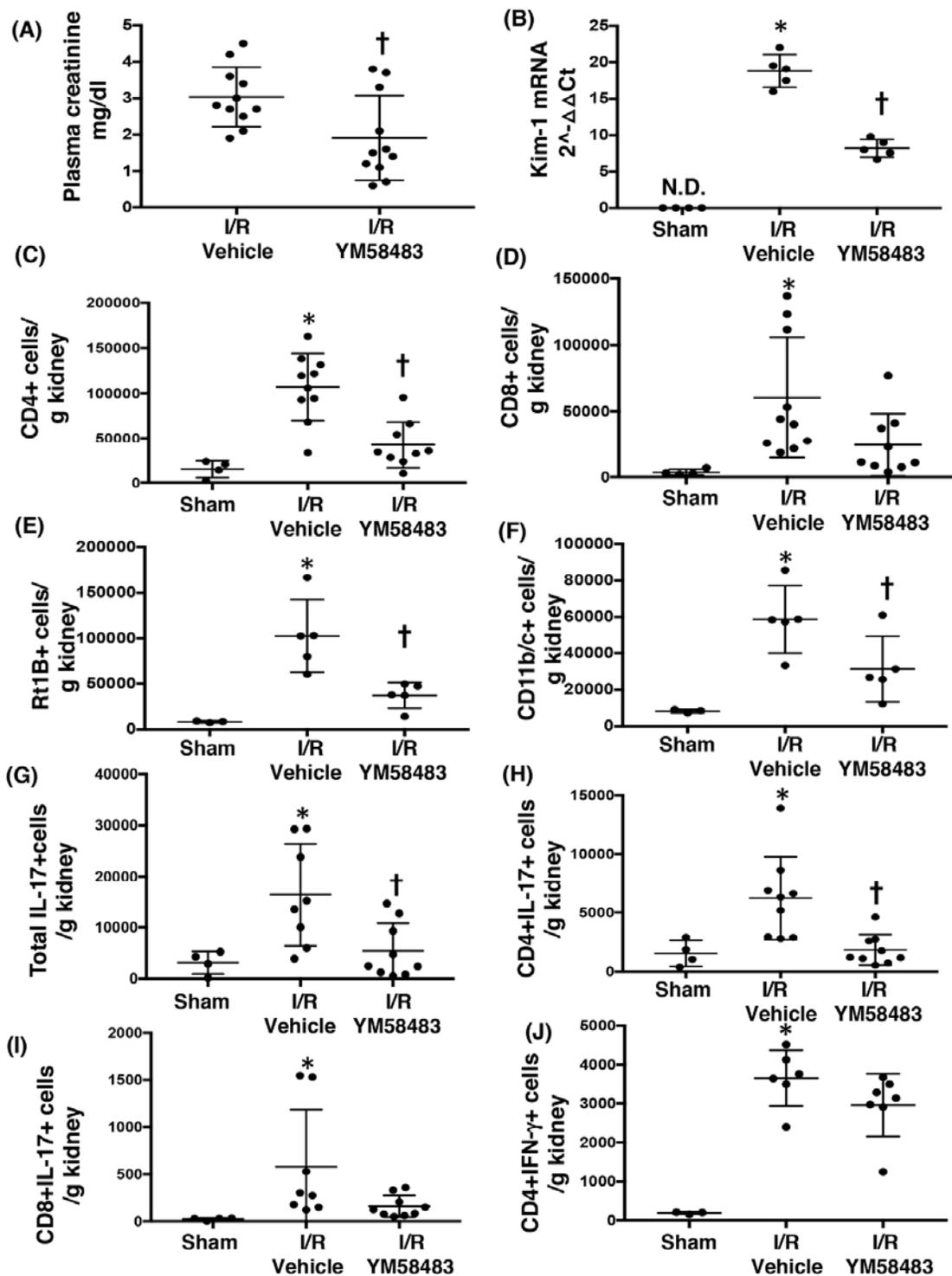
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**Figure 1: Co-expression of Orai1 and IL17 in CD4+ T cells following renal I/R** A) Left; Contour plot of renal lymphocytes stained with antibodies for CD4 and IL17 to identify Th17 cells. Orai1 staining in gated Th17 cells of sham is shown in middle panel and 2 days following I/R in the right panel. B) The number of Orai1+/CD4+ cells in sham and post-I/R rat kidneys is shown. C) The number of Orai1+/CD4+/IL17+ cells in sham and post-I/R rat kidneys is shown. D) Representative histogram of Orai1+ cells in kidney CD4 fraction (left) 2 days following I/R and the distribution of IL17 expression as a function of Orai1 is shown on right. E) The percentage IL17+ cells as a function of Orai-1 expression in CD4+ cells following sham surgery or 2 days following I/R is shown F) Sustained expression of Orai1 in CD4+ cells following 7 days of recovery from I/R surgery is shown as representative histogram (left) and total number of Orai1+/CD4 cells is shown in the right panel. In B, C, E and F, data are mean  $\pm$  SE of 4-5 rats per group; \* indicates  $P < 0.05$  sham vs post-AKI by Student's t-test. In panel E, \* indicates  $P < 0.05$  in sham vs. I/R, † indicates  $P < 0.05$  in Orai1- vs Orai1+ cells, by one-way ANOVA and Tukey's post-hoc test.

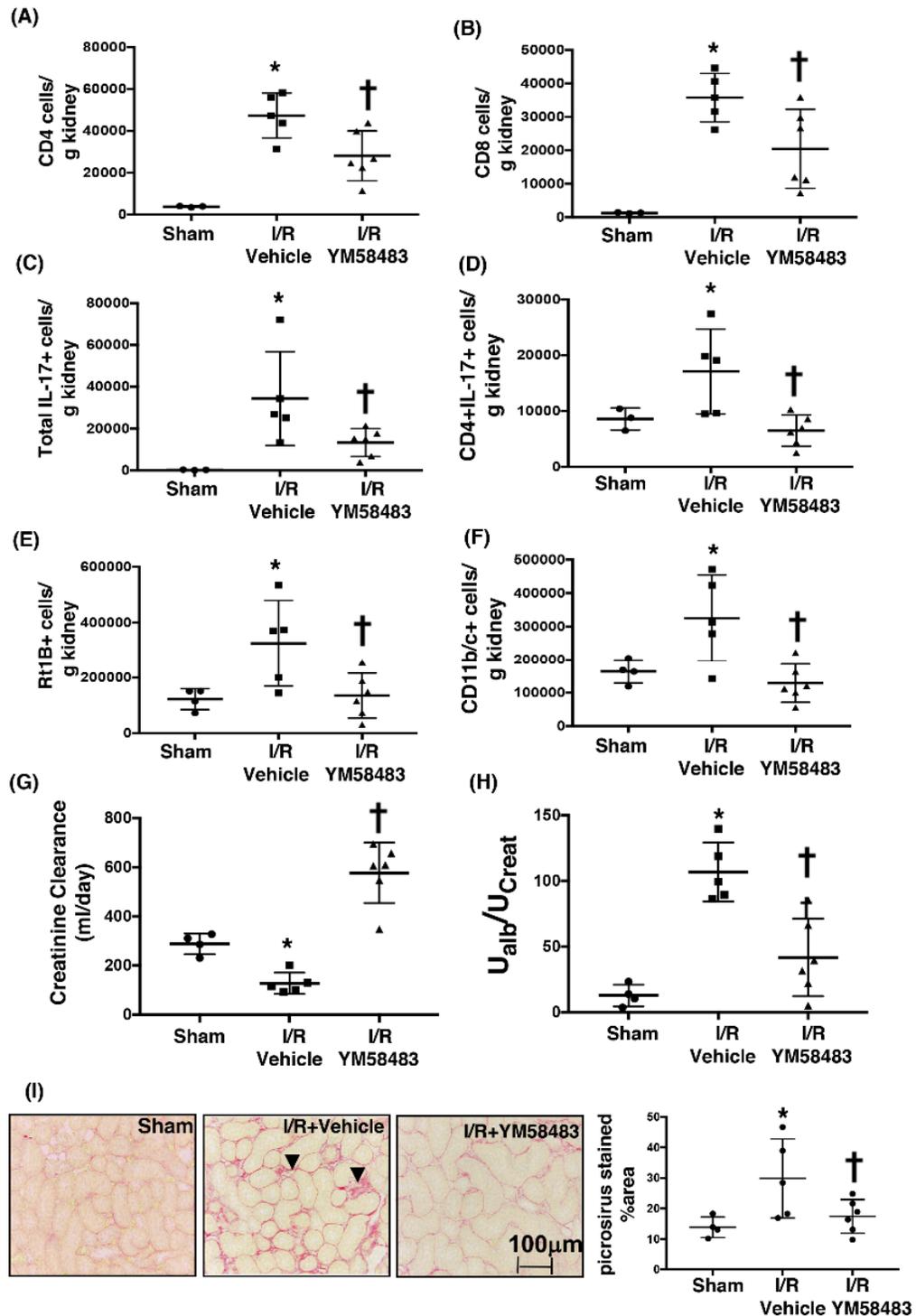


**Figure 2: Orai1 activity contributes to IL17 expression in CD4+ lymphocytes primed by renal ischemia/reperfusion injury.** A) Illustrates representative FACS showing increased IL-17 expression in CD4+ cells from 7 day post-AKI rats following stimulation in vitro with 170 mM Na<sup>+</sup> and Ang II vs. control media; B) percent IL17+ cells in CD4+ cells isolated 7 days following sham or AKI and stimulated in vitro; C) IL17 mRNA, expressed as  $2^{-\Delta\Delta CT}$  of kidney derived CD4+ cells isolated 7 days following I/R surgery and stimulated in vitro. In panel B and C, control refers to AKI-primed CD4+ cells stimulated with 170 mM Na<sup>+</sup> and Ang II ( $10^{-7}M$ ), and SOCE inhibitors included as labeled. D) Fura-2 fluorescence imaging of intracellular Ca<sup>2+</sup> in CD4+ lymphocytes in response to increased Na<sup>+</sup> (170 mM) plus Ang II ( $10^{-7}M$ ), as indicated in the timeline and expressed as the ratio of fluorescence using 340/380 nm excitation. Shown are representative tracings of CD4+ cells from kidney following sham surgery (black) or I/R injury (red), or from I/R injury with co-incubation with AnCoA4 (blue). The inset illustrates representative visual field of multiple fura-2 loaded cells. E) Percent of cells manifesting an increase Ca<sup>2+</sup> response relative to baseline following in vitro stimulation with increased Na/Ang II. Data are mean  $\pm$  SE from 4-5 rats per group per assay; \* indicates  $P < 0.05$  vs unstimulated cells (i.e., no Ang II and normal Na, data not shown, see Supplemental Figure 3); † indicates  $P < 0.05$  inhibitors vs stimulated post-AKI cells by one-ANOVA and Tukey's post-hoc test.

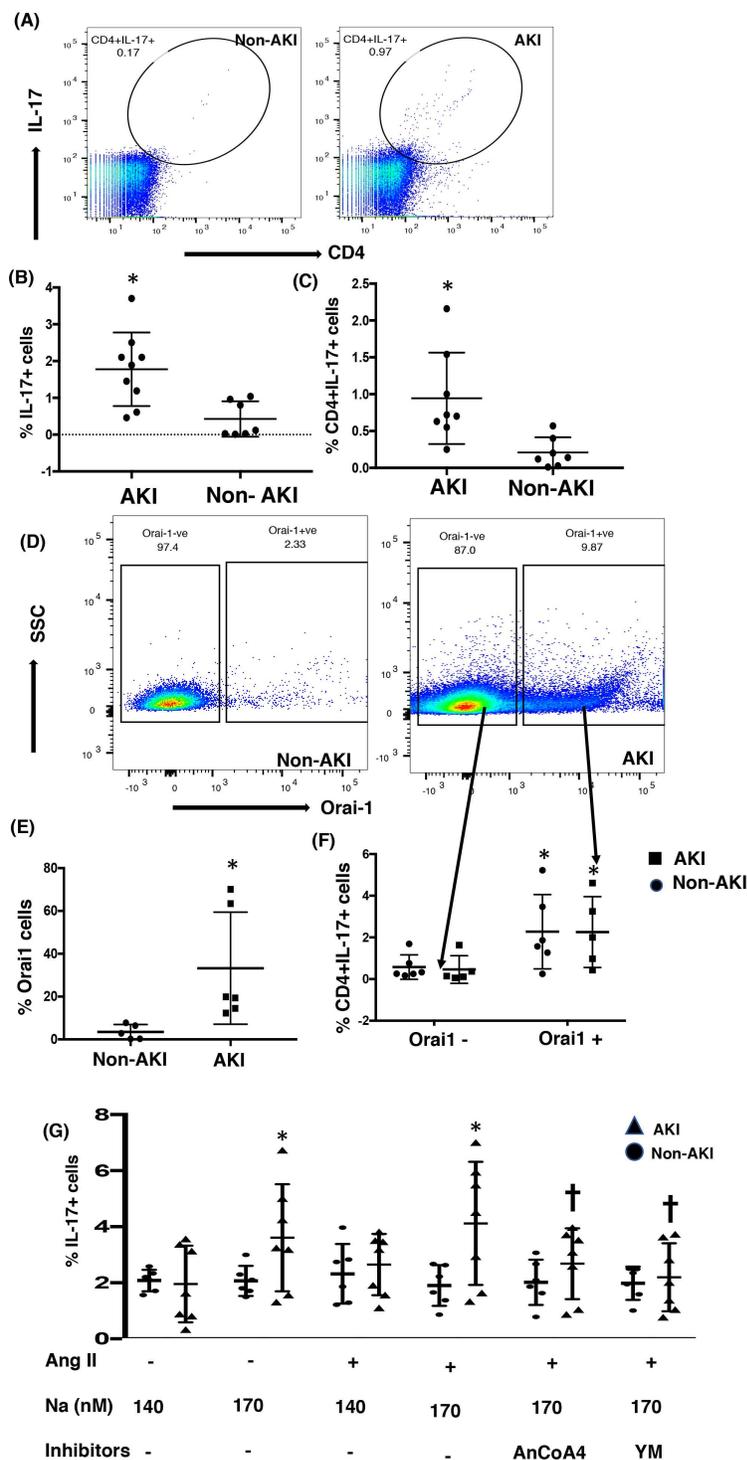


**Figure 3: YM58483 pretreatment attenuates renal ischemia reperfusion injury.**

**A)** Assessment of renal function by plasma creatinine 24 hours following I/R in Sprague-Dawley rats pretreated with YM-58483 or vehicle. Values for sham rats were  $0.4 \pm 0.1$  mg/dl are not shown on graph. **B)** Kim-1 mRNA levels from total kidney RNA 24 hours following sham or I/R are shown, (N.D. = not detectable). Total kidney derived CD4+ and CD8+ cells are shown in C and D, respectively; total B cells (RT1B+) and dendritic/macrophage cells (DC/M $\phi$ ; CD11b/c+) are shown in E and F, respectively. The total number IL17+, CD4+/IL17+ and CD8+/IL17+ cells are shown in G, H, I respectively. CD4+/IFN $\gamma$ + cells are shown in J. Data are mean  $\pm$  S.E. (N=11rats/group in panel A and 7-8 rats in B-J). \*P<0.05 I/R vehicle vs sham; † P<0.05 vehicle vs YM58483 by one way ANOVA and Tukey's post-hoc test.



**Figure 4. YM58483 attenuates inflammation and progressive renal damage following I/R and exposure to high salt diet.** Shown are levels of renal CD4+ (A), CD8+ T cells (B), IL-17+ expressing cells (C), and CD4+/IL17+ cells (D). Levels of B cells (E) and dendritic cells/M $\phi$  (F) are shown. Creatinine clearance from 24 hour urine collections at day 62-63 is shown in panel G and urinary albumin/creatinine ratio is shown in panel H. Panel I illustrates representative picosirius red stained sections through renal outer medulla from sham, I/R vehicle or I/R + YM 58483-treated rats. Quantification of stained area is illustrated in J. Data are mean  $\pm$  S.E. (N=5-6 rats/group). \*P<0.05 I/R vehicle vs sham; †P<0.05 vehicle vs YM58483 by one way ANOVA and Tukey's post-hoc test.



**Figure 5. Elevated Th17 and Orai1 expression and effect of SOCE on IL17 responses in CD4+ blood lymphocytes in critical ill patients with vs without AKI.**

A) Representative FACS analysis of total peripheral blood cells in non-AKI (left, n=8) vs AKI patients (right, n=9) demonstrating the increase in CD4+IL17+ expressing cells. Quantification of the percentages of total IL17+ cells (B) and CD4+IL17+ cells (C) in AKI vs non-AKI patients are shown. Representative dot plot showing an increase in Orai-1+ cells in AKI patients as compared to non-AKI patients is shown in (D) and the percentage of Orai-1+ cells in AKI vs non-AKI patients is shown in (E). (F) The %CD4+IL17+ gated on Orai1+ and Orai1- fraction (arrows) is shown. In panel G, IL17 response to stimulation with elevated sodium (170 mM) and Ang II is shown for CD4+ cells isolated from AKI (n=5) or non-AKI (n=5) patients. Data are expressed as mean  $\pm$  S.E. In panel B, C and D \* indicates P<0.05 in AKI vs non-AKI patients by Student's t-test. In panel F, \* indicates p<0.05 in Orai1+ vs Orai1- cells. In G, \* indicates p<0.05 vs unstimulated (i.e., 140 mM and no Ang II), † p<0.05 inhibitors vs stimulated using one-way ANOVA and Tukey's post-hoc test.