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## Heterogeneity of epigenetic changes at ischemia/reperfusion- and endotoxin-induced acute kidney injury genes

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### Abstract

Aberrant gene expression is a molecular hallmark of acute kidney injury (AKI). Since epigenetic processes control gene expression in a cell- and environment-defined manner, understanding the epigenetic pathways that regulate genes altered by AKI may open vital new insights into the complexities of disease pathogenesis and identify possible therapeutic targets. Here we used matrix chromatin immunoprecipitation and integrative analysis to study twenty key permissive and repressive epigenetic histone marks at transcriptionally induced *Tnf*, *Ngal*, *Kim-1* and *Icam-1* genes in mouse models of AKI; unilateral renal ischemia/reperfusion, lipopolysaccharide (LPS) and their synergistically injurious combination. Results revealed unexpected heterogeneity of transcriptional and epigenetic responses. *Tnf* and *Ngal* were transcriptionally upregulated in response to both treatments individually, and to combination treatment. *Kim-1* was induced by ischemia/reperfusion and *Icam-1* by LPS only. Epigenetic alterations at these genes exhibited distinct time-dependent changes that shared some similarities, such as reduction in repressive histone modifications, but also had major ischemia/reperfusion vs. endotoxin differences. Thus, diversity of changes at AKI genes in response to different insults indicates involvement of several epigenetic pathways. This could be exploited pharmacologically through rational-drug design to alter the course and improve clinical outcomes of this syndrome.

### Keywords

acute kidney injury; gene expression; ischemia reperfusion; sepsis

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## Introduction

It is estimated that 2 million people worldwide die from acute kidney injury (AKI) annually, equaling the number of deaths from AIDS<sup>1-2</sup>. Moreover, AKI is becoming an ever greater health care burden as its incidence rises at alarming rates, placing patients at risk for chronic kidney disease (CKD) and increasing the likelihood of end stage renal disease<sup>1, 3-7</sup>. Cellular and molecular basis of AKI pathogenesis were examined in multiple studies that brought better understanding of the syndrome's pathophysiology. However treatment of AKI has remained mainly supportive and available therapeutic modalities show only minimal improvement of the high mortality associated with this disease over the last fifty years<sup>8-9</sup>. Historically, mechanistic studies in AKI have focused on single factors or pathways. Although informative these approaches fail to capture the system-wide complexity and heterogeneity of AKI and have thus yielded limited translational and clinical breakthroughs. These considerations suggest that novel integrative approaches are needed, rather than those focused on a single gene or pathway<sup>10</sup>, to study the temporally dynamic and functionally diverse course of AKI.

Aberrant gene expression is a molecular hallmark of AKI<sup>11</sup> and several gene products of renal injury, such as KIM-1 and NGAL, have been implicated in the syndrome and used as AKI biomarkers<sup>10, 12</sup>. Still, the molecular mechanism mediating detrimental gene expression alterations are not well understood. Epigenetic processes control gene expression in a cell- and environment-defined manner. Epigenetic modifications of DNA and histones have been viewed as either transcriptionally permissive or repressive<sup>13-14</sup>. As such, epigenetic marks can serve as sensitive indicators of transcriptional and other changes at a gene. Recent advances in transcription and epigenetic technologies allow not only measurements of rates of transcription and chromatin changes but also epigenetic modifiers bound to chromatin<sup>15-18</sup>. An important translational application of identifying the chromatin modifiers bound to disease-causing genes is discovery of novel epigenetic targets for potential rational-design drug interventions to ameliorate renal injury.

In the clinical setting, diverse insults contribute to AKI and activate heterogeneous pathways that<sup>10</sup> can interact synergistically to exacerbate injury<sup>19-20</sup>. Understanding the epigenetic landscape of genes induced by AKI may open fundamentally new insights into disease pathogenesis advancing rational-design epigenetic-based therapies. Ischemia-reperfusion (I/R) and sepsis are among the most common causes of AKI<sup>4</sup>, but represent two clinically and pathophysiologically distinct entities<sup>21-25</sup>. The mouse model of endotoxemia in the setting of preceding unilateral renal ischemia/reperfusion (I/R) captures the molecular events of multifactorial AKI with sepsis being a component<sup>15, 26-27</sup>. In this model, the proximal tubular cells transcriptionally hyper-respond to endotoxin (lipopolysaccharide, LPS) leading to exaggerated renal cytokine production<sup>15, 28</sup>.

We applied high throughput RT-qPCR and matrix chromatin immunoprecipitation (ChIP) platforms<sup>29-30</sup> to systematically map the heterogeneity of transcriptional and epigenetic responses in AKI caused by LPS, I/R, and their combination (LPS+I/R) in time course animal experiments.

## Results

In the current study we utilized a model of endotoxin hyperresponsiveness in the setting of I/R (Fig. S1) to examine several issues associated with heterogeneity of transcriptional and epigenetic responses in AKI. Specifically, we asked: i) Which AKI-related genes exhibit endotoxin transcriptional hyperresponsiveness in the setting of I/R (i.e., a synergistic effect of I/R+LPS)? ii) Which of the established AKI biomarker mRNAs report endotoxin hyperresponse in the setting of I/R? iii) What is the time course of the endotoxin transcriptional hyperresponse for different genes? iv) What are the similarities and differences in epigenetic alterations associated with transcriptional responses in a group of representative I/R and endotoxin-induced genes?

### AKI is characterized by heterogeneous mRNA responses

A high throughput RT-qPCR microplate platform was used to assess mRNA levels of a panel of large number of genes previously implicated in AKI including several biomarkers (Ngal, Kim-1, Klotho, Netrin1, IL-18, L-FABP, Timp2, angiotensinogen (Agt) and Igfbp7)<sup>10, 31-32</sup>. Cluster analysis of gene expression profiles demonstrated highly dynamic patterns across time points and types of injury (Fig. 1). While the AKI-associated genes could be broadly divided into those that were up or down-regulated during injury, a closer inspection revealed significant diversity in transcriptional responses.

Tnf<sup>15</sup> and several co-clustered genes (Vcam1, Pai1, Tlr2 and Ngal) exhibited a robust endotoxin response with synergistic effects in I/R+LPS kidneys (Fig. 1). In contrast, several genes including Kim-1 and Tlr4 were up-regulated following I/R but not LPS treatment, whereas the expression of Icam1 was up-regulated by LPS alone with minimal effect of I/R. Many genes including Klotho, Netrin1, and Agt<sup>32</sup>, were characterized by down-regulated mRNA expression following I/R (26-74hrs) but remained unresponsive to LPS. In contrast, the expression levels of several angiogenic genes including Tek, Pecam-1 and Kdr<sup>33</sup>, were decreased in response to LPS but they exhibited minimal response to I/R. Notably, several mRNAs whose products are being introduced as novel AKI biomarkers including IL-18, L-FABP, Timp2 and Igfbp7 changed little or not all in our AKI model. The discrepancies between the current results and the AKI biomarker literature<sup>10, 34</sup> may reflect species differences and/or the fact that here we examined mRNA and not protein levels. Nonetheless, our results in this mouse model suggest that no single biomarker can capture the heterogeneity of AKI transcriptional responses. For example, septic AKI signature (Icam-1 expressed in the endothelium) can be very different from I/R (Kim-1 expressed in the proximal tubules). Thus, measurements of combinatorial biomarker may be more useful to encompass responses from various injured renal tissue compartments.

Epigenetic biomarkers and therapies are emerging as important new means to diagnose and treat disease. Given their well-documented role in the pathogenesis of renal injury, and distinct temporal and injury-specific expression patterns in our animal model of AKI (Fig. 2), we chose Tnf, Ngal, Kim-1 and Icam-1 as representative AKI induced genes for in-depth epigenetic analyses.

## Pol II density measurement at *Tnf*, *Kim-1*, *Ngal* and *Icam-1* genes confirms that transcription plays a role in their induced expression during AKI

Measurement of Pol II levels at gene loci provides means to assess rates of transcription<sup>35</sup>. We analyzed Pol II binding at *Tnf*, *Kim-1*, *Ngal* and *Icam-1* using an antibody that recognizes both unphosphorylated and phosphorylated C-terminal domain CTD (4H8), and observed that changes in the levels of Pol II at genes generally matched mRNA responses (compare Fig.2 and 3). Specifically, we found that: i) there was endotoxin-induced Pol II hyper-recruitment in the setting of renal I/R at the *Tnf* and *Ngal* genes, ii) Pol II levels at *Kim-1* gene were increased in response to I/R but not to LPS, and iii) at the *Icam-1* locus Pol II responded to LPS but not I/R. Similar results were obtained with Pol II antibodies that recognize either phosphorylated (pSer2 and pSer5) or unphosphorylated (8WG16) CTD (Supplemental Figs. S2 and S3). Of note, Pol II induction was seen 3hrs post I/R, at *Tnf*, *Kim-1* and *Ngal*, a time point when mRNA changes were not yet detectable (Fig.3). Taken together, these results indicate that upregulation of mRNA of these selected genes during I/R, LPS and I/R + LPS are, at least in part, transcriptionally mediated. Next we used a panel of antibodies to permissive and repressive histone marks to systematically compare epigenetic profiles of *Tnf*, *Kim-1*, *Ngal* and *Icam-1* in this AKI model.

### Epigenetic patterns of permissive marks in AKI

Histone acetylation can increase in response to extracellular signals and generate a transcriptionally permissive chromatin structure<sup>30, 36</sup>. Histones acetylated at lysine residues also serve as docking sites for recruitment of chromatin modifiers. H3K9Ac is one of the most studied permissive histone modifications. H3K9Ac levels increased at *Tnf* post I/R (26 and 74hrs) without endotoxin hyper-response (Fig.4). Given that *Ngal* Pol II profile was similar to *Tnf* (Fig.3), it was surprising that no increases in H3K9Ac were detected at *Ngal* (Fig.4). Furthermore, there was only a small increase in H3K9Ac at the I/R-responsive *Kim-1* locus and no H3K9Ac changes at *Icam-1* even though there was robust Pol II response to LPS (compare Figs.2 and 3). Three other acetylation marks, H3K18Ac, H3K27Ac and H4K5/8/12/16Ac also exhibited changes at *Tnf* in response to both I/R and LPS (Figs. S4-6). Given that these measurements demonstrated robust H3K9Ac, H3K18Ac, H3K27Ac and H4K5/8/12/16Ac 7 changes at *Tnf* but little or no changes at all other genes (Figs.4, S4-6), we concluded that up-regulation of *Icam-1*, *Ngal*, and *Kim-1* occurred without changes in their histone acetylation. Nonetheless, other acetylation events that were not examined in our study may have contributed to the robust AKI-induced changes in the expression of these genes.

Unlike acetylation, methylation does not change lysine charge, and alters transcription by providing docking sites for chromatin modifiers. Methylation of histone H3 lysine 4 (H3K4m1, H3K4m2 and H3K4m3) marks chromatin states of active transcription<sup>14</sup>. We observed I/R-induced increases in H3K4m3 levels at *Tnf* at 26 and 74hrs but not at 3hrs (Fig.5). There was little or no response in H3K4m3 with LPS and there was no endotoxin hyper-response in the setting of I/R at this locus. H3K4m2 alterations at *Tnf* exhibited a similar pattern (Fig. S7). H3K4m3 changes at the *Kim-1* and *Ngal* genes were similar to those seen at the *Tnf* locus but at a smaller magnitude. At the *Icam-1* locus, we did not detect any H3K4m3 changes despite robust LPS-induced transcriptional activation of this

gene (Fig.3). Permissive H3 serine 10 phosphorylation (H3pSer10) was also responsive to I/R but not LPS (Fig. S8). These results illustrate that epigenetic responses to I/R are distinct from those induced by endotoxin.

### Epigenetic patterns of elongation marks in AKI

Higher levels of H3K36m3 have been correlated with enhanced transcription elongation rates<sup>37</sup>. At the *Tnf* locus both I/R and LPS increased H3K36m3 levels and there was a synergistic effect for I/R+LPS at 26 and 74 hrs (Fig.6). At the *Kim-1* locus, H3K36m3 increased following I/R but not LPS, and the increase was far greater at 26 and 74hrs compared to 3hrs. There was small H3K36m3 increase at 3 and 26hrs post I/R at *Ngal* and there was LPS hyper-response at 26hrs post I/R. Levels of H3K36m3 at *Icam-1* increased in response to either I/R and LPS and the effects of I/R + LPS appeared additive. Changes in H3K36m2 were similarly responsive to either I/R or LPS (Fig.S9). H3K36m2 and H3K36m3 changes, at least in part, resembled Pol II profile at these loci (Fig.3) suggesting that they may play a role in increased transcription of these genes in response to I/R and LPS. Di-methylation of the histone H3 lysine 79 (H3K79m2), found within the nucleosome core, is also thought to be associated with transcription elongation. H3K79m2 generally increased 3 and 26hrs after I/R at all four genes. LPS alone did not have any effects on this mark (Fig.S10). Thus, overall the H3K79m2 responses were different compared to H3K36m3 and H3K36m2.

### Epigenetic patterns of repressive marks in AKI

We examined repressive modifications representing each of the core histones, H3K27m3, H3K9m2, macroH2A, H2AK119ub1, H2BK120ub1 and H4pSer1. I/R decreased H3K27m3 at *Tnf* within 24 and 74 hrs but not after 3hrs (Fig.S11). Similarly, late I/R H3K27m3 decreases seen at *Kim-1*, *Ngal*, and *Icam-1*. Late (74hrs) post I/R reductions also seen in H3K9m2 levels at *Kim-1* (Fig.S12). LPS reduced H3K27m3 and H3K9m2 levels only at *Icam-1* (Fig.S11-S12). Given that LPS reduced both H3K27m3 and H3K9m2 at the *Icam-1* gene these modification may play a role in induction of this gene in response to endotoxin. MacroH2A, H2AK119ub1 and H2BK120ub1 were more responsive to I/R than LPS (Figs. S13-S15). Interestingly, H4pSer1<sup>38</sup> exhibited a bi-phasic I/R response at all four genes with increase at 3hrs and decrease at 74hrs post I/R (Fig.7).

### Integration analysis of mRNA, Pol II and histone modifications underscores diversity of epigenetic portraits at *Tnf*, *Ngal*, *Kim-1* and *Icam-1* loci in AKI

Genome-wide mapping has revealed a host of epigenetic ground-states characterized by clusters of histone modifications that coexist to control chromatin structure and function<sup>39-40</sup>. However, little is known on how sets of functionally related epigenetic modifications respond to injury from their baseline states. To obtain an overview of the transcriptional and epigenetic signatures in AKI, we integrated mRNA, Pol II and histone modifications and summarized our findings using a heatmap (Fig. 8 and Fig.S16). Log-transformed I/R, LPS and I/R+LPS mean values normalized to the control groups of mice were used to generate gene portraits (Figs.8 and S16). The heatmap illustrates concordance between mRNA and Pol II levels indicating that, at least in part, expression of these genes in response to renal injury is transcriptionally mediated. *Tnf* and *Ngal*, two genes that exhibited

synergistic interaction between I/R and endotoxin treatments had more similar profiles compared to Kim-1 and Icam-1. Still, the integrative analysis revealed a notable lack of injury-induced hyperacetylation at Ngal that was clearly observed at Tnf. Further, comparing Kim-1 and Icam-1 portraits underscored the striking differences between these two genes. Despite heterogeneity among AKI-induced genes, there was a general trend for the repressive marks to fall at 26 and 74hrs post I/R, implicating decreased silencing marks as a mechanism for increased transcription in all four genes.

## Discussion

This study represents an in-depth exploration of the epigenetic alterations at several key AKI-associated genes. We found that two commonly used models of kidney injury, I/R and LPS, have strikingly different effects on expression and epigenetic profiles of these candidate genes in a time dependent manner.

Previous experimental AKI studies have shown a host of permissive epigenetic changes at the transcriptionally induced Tnf gene <sup>2, 15, 41</sup>. Much less is known about transcriptional and epigenetic control of Ngal, Kim-1 and Icam-1 genes in AKI. Here we found that the expression pattern of these and other AKI-related genes in response to I/R and endotoxin induced renal injury are different.

Some of the observed transcriptional heterogeneity may be attributable to cell type specific expression of AKI-associated genes. For example, Icam-1 is preferentially expressed in endothelial cells <sup>42</sup> thus the distinct mRNA response of this gene compared to the others (Fig.2) might represent cell type-specific mechanisms. In contrast, Kim-1, like Tnf and Ngal, is primarily expressed in the proximal tubules so the absence of Kim-1 response to LPS at the time point examined (Fig.2) suggests that the pathways that control its expression in this cell type are different from those of the other two genes <sup>43</sup>.

In our model system, diversity in the transcriptional responses of Tnf, Ngal, Kim-1 and Icam-1 to I/R and LPS were further highlighted by differences in epigenetic alterations at these loci (Fig.8 and S16). For example, Pol II recruitment at both Tnf and Ngal closely followed changes in mRNA levels (Fig.3) but unlike induced histone acetylation at Tnf, there was little or no change in histone acetylation at the Ngal gene at the time-points examined (Fig. 8 and S16). Similarly, Kim-1 and Icam1 exhibited more modest increases in histone acetylation compared to the robust Tnf response. These results demonstrate that transcription could be upregulated with or without increased histone acetylation in a gene-specific manner. Our findings in AKI are consistent with similar observations in other systems, for example, during erythroid differentiation, GATA factor-induced transcription occurs while permissive chromatin modifications remain unchanged <sup>44</sup>. In contrast to permissive histone acetylation processes, there was greater AKI-induced reduction in repressive marks at Ngal, Kim-1 and Icam-1 compared to Tnf gene (Fig.8 and S16), suggesting an alternative mechanism for the observed upregulation of these three genes. Our integrative analysis encompassing multiple injury models, temporal profiling, and detailed epigenetic interrogation illustrates the rich diversity of transcriptional control in AKI, and



highlights common and major differences in regulation of gene expression in this complex syndrome (Fig.8 and S16).

There are several limitations in the current study. We assessed only one time point post endotoxin injection and therefore did not capture potential changes in LPS-induced Kim-1 expression later in the course of evolving injury. Similarly if we had examined more time points post I/R we may have noted additional temporal changes at the Icam-1 locus. Although we investigated twenty different transcription and epigenetic events—a more comprehensive survey than any previous AKI epigenetics study—the number of known epigenetic marks is significantly larger than our examined set. Further, we studied only a select group of candidate AKI genes and not a genome-wide screen (Fig.1). These limitations reflect remaining technical challenges preparing large number of renal cortex samples for ChIP assays. It is anticipated that with further advancement in epigenetic platforms combined with next generation sequencing, these technical bottlenecks can be overcome. In this context, our general approach can serve as ground-work for future AKI studies using the next generation epigenetic technologies.

How does the observed epigenetic heterogeneity in AKI advance research and ultimately help us improve clinical outcomes of this syndrome? First, these studies provide further evidence that epigenetic mechanisms play a key role in AKI<sup>2</sup>. Second, the rapid decline of renal function in AKI followed by structural changes<sup>9</sup> poses a challenge in initially identifying the culprits of injury and then instituting appropriately-timed therapies tailored to specific patients. Our study design allowed us to demonstrate that several epigenetic alterations at different genes exhibited distinct time-dependent changes following AKI (e.g. H4pSer1). Thus, extending our integrated approach to urine nucleic acids and chromatin analysis<sup>45-47</sup> may help identify appropriate time windows for AKI treatment. Third, epigenetic modifications are reversible, providing means for pharmacologic manipulation of gene expression. Therefore, epigenetic enzymes mediating detrimental changes in transcription can be targeted to modulate the severity of renal injury.

## Conclusion

The striking diversity of epigenetic portraits of I/R- and endotoxin-induced renal genes observed in this study suggests that different signaling pathways drive recruitment of distinct sets of epigenetic enzymes to modify chromatin structures of candidate AKI genes. Thus, these observations open up an exciting possibility that this epigenetic heterogeneity can be exploited to devise therapeutic interventions to target groups of AKI-associated genes with similar epigenetic profiles, while minimizing off target side effects.

## Methods

**Reagents**—Bovine serum albumin (BSA), phosphate buffered saline (PBS), salmon sperm DNA, and protein A were from Sigma, and proteinase K was from Invitrogen. Matrix ChIP-MeDIP 96-well polypropylene plates were from Bioexpress. Formaldehyde, ethanol, NaCl, EDTA, Triton X-100, NP-40, Tris, leupeptin, PMSF, p-nitrophenyl phosphate, NaF,

Na<sub>3</sub>VO<sub>4</sub>, Na<sub>2</sub>MoO<sub>4</sub> and β-glycerophosphate were from Sigma. The antibodies were commercially available and are listed in Table 1.

**Animal model of AKI**—All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Washington and Fred Hutchinson Cancer Research Center. CD 1 mice (Charles River Laboratories, Wilmington, MA; 6-10 weeks of age, male; 30-35 gms), were maintained under routine vivarium conditions. The experimental protocol in this study is shown in Fig.S1. To study mechanisms of renal endotoxin hyperresponsiveness, we have used an I/R model<sup>27</sup> where one kidney of each animal is subjected to ischemia/reperfusion with subsequent treatment with either saline (I/R) or LPS (I/R+LPS). In brief, mice were anesthetized with pentobarbital and subjected to a midline abdominal incision under sterile conditions. Left renal ischemia was induced with an atraumatic microvascular clamp applied to the renal pedicle. After 30 min of unilateral renal artery occlusion, the clamp was released and reperfusion of the entire kidney was assessed visually (by loss of global cyanosis). A 30 min ischemic insult was selected for study because it induces moderately severe ischemic kidney damage<sup>48</sup>. One, twenty-four or seventy-two hours after I/R injury, mice received a tail vein injection of either LPS (2 mg/kg; 0111:B4; 1-2630; Sigma, St. Louis, MO; in 80 μl of saline) or saline. Two hours after injection, mice were re-anesthetized, the abdominal cavity was opened, the kidneys were extracted, and renal cortical samples were cut from the kidneys with a razor blade and rapidly frozen at -70°C. The mice died within 30 sec due to exsanguinations. Thus, the timing of sacrifice relative to I/R was 3, 26 and 74 hrs, and 2 hrs post either LPS or saline treatment. As previously documented<sup>28</sup> the right non-ischemic, contralateral (control) kidney recapitulates what is seen in sham operated kidneys and hence served as an internal control. Histology for I/R and LPS models of AKI have previously been evaluated<sup>48-50</sup>

**RNA Extraction and cDNA Synthesis**—RNA was extracted from tissue fragments using Trizol reagent as per the manufacturer's protocol. To synthesize cDNA, 400 ng of Trizol-extracted total RNA was reverse transcribed with 200 units MMLV reverse transcriptase (Invitrogen) and oligo dT primers in 10μl reactions in 96-well microplates. RT reactions were diluted 100-fold prior to running qPCR<sup>51</sup>.

### Chromatin preparation and multiplex Matrix ChIP platform

The multiplex microplate Matrix ChIP method was previously described<sup>29-30</sup>. Briefly, for ChIP assays, tissue fragments (10-20 mg) were cross-linked with formaldehyde, and chromatin was sheared using Diagenode Bioruptor. ChIP assays were done using protein A-coated 96-well polypropylene microplates as described before<sup>29</sup>. 1-2 μl of eluted DNA was used in 2-4 μl real-time PCR reactions (ABI7900HT). All PCR reactions were run in quadruplicates. PCR calibration curves were generated for each primer pair from a dilution series of total mouse genomic DNA. The PCR primer efficiency curve was fit to cycle threshold (Ct) versus log(DNA concentration) using an r-squared best fit. DNA concentration values for each ChIP and input DNA sample were calculated from their respective average Ct values. Final results are expressed as fraction of input DNA<sup>30</sup>. Matrix ChIP PCR primers to 5' end of genes used in this study are shown in Table S2 and list of antibodies in Table S3 (supplement).



## Statistics and visualization

To acquire, store and analyze large data sets generated by the high throughput Matrix ChIP platform, we developed a novel graphical method (GraphGrid). Pair-wise statistically significant differences are represented by the size of a circle for each comparison made with a small circle representing  $p < 0.05$ , a large circle indicating  $p < 0.01$  and no circle implying non-significance. GraphGrid uses a two-tailed Student's t-test to compute p-values<sup>15</sup>.

**Cluster analysis**—All RT-qPCR gene expression values during injury conditions (I/R, LPS, I/R+LPS) were normalized to their uninjured, saline-treated control values and log-transformed. PCR primers are listed in Table S1 (supplement). We applied hierarchical clustering to log-transformed relative expression values using Euclidian distance measure and grouped genes based on their expression patterns across injury conditions<sup>52</sup>. Heatmaps were also generated using similarly normalized and log-transformed values for Pol II levels and the various epigenetic marks under different AKI conditions.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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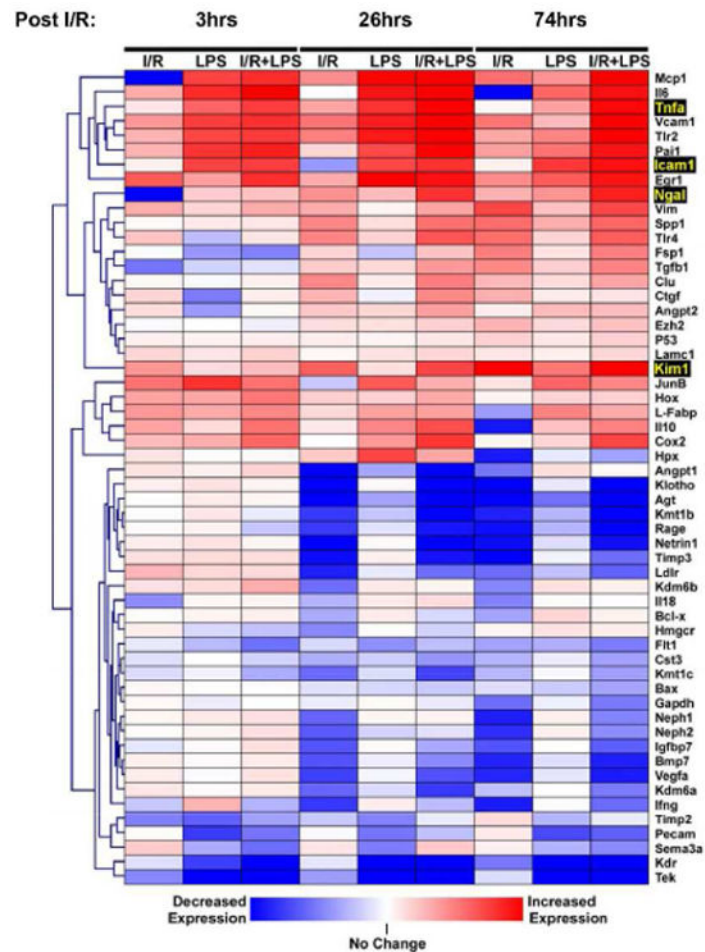
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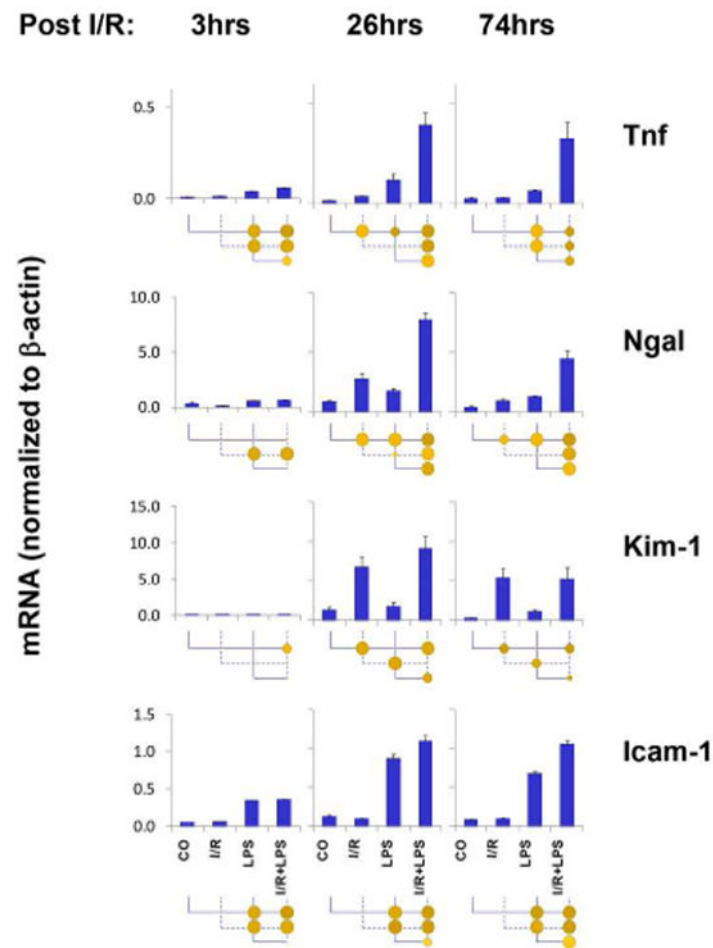
### Key points of the study

- Different but overlapping groups of genes respond to I/R and endotoxin treatments in kidneys.
- Transcription plays a role in AKI-induced Tnf (I/R and endotoxin), Ngal (I/R and endotoxin), Kim-1 (I/R) and Icam-1 (endotoxin) expression.
- Epigenetic changes at AKI genes share similarities but also have major differences.
- Epigenetic alterations at different genes exhibit distinct time-dependent changes.
- Heterogeneity of epigenetic changes at AKI genes indicate involvement of several epigenetic pathways that could potentially be exploited pharmacologically through rational-drug design to alter the course and improve clinical outcomes of this syndrome.



**Fig.1. Cluster analysis of temporal gene expression profiles in AKI**

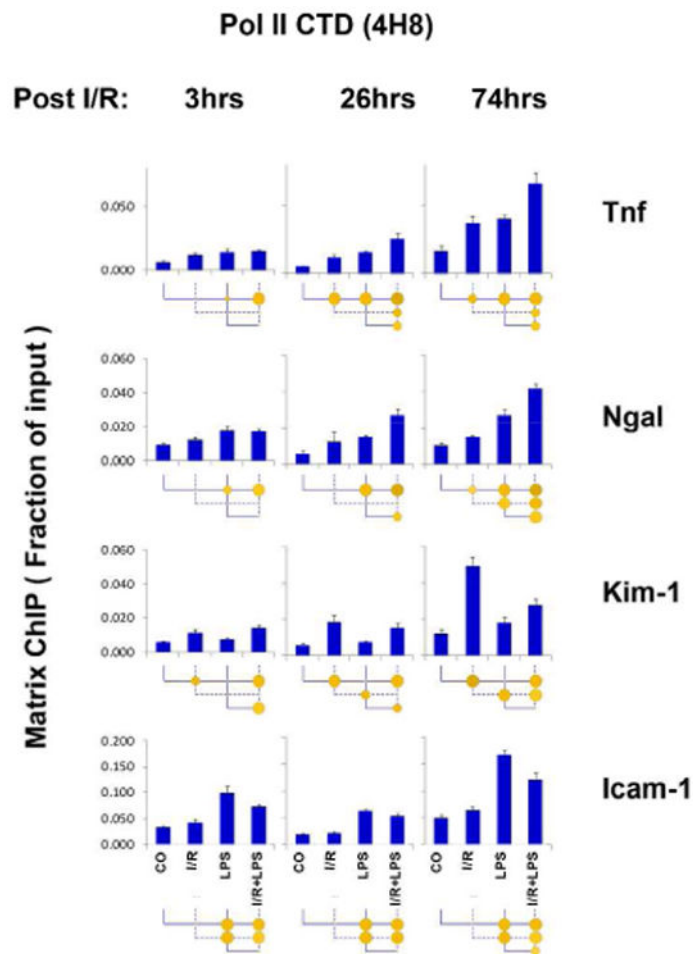
The timing of harvesting the kidneys post I/R injury was 3, 26 and 76 hrs, and 2 hrs after either LPS or saline treatment (Methods). Total RNA from mice renal cortex was used in RT reactions with oligo dT. cDNA was used in real time PCR (qPCR) with gene specific primers (Table S1, supplement). mRNA level of a given gene in each sample was normalized to  $\beta$ -actin transcript. The mRNA levels of 56 AKI-associated genes were measured using and clustered based on expression pattern differences across time and injury model. Note the diverse transcriptional responses to injury of co-clustered genes as highlighted in the figure. Data are represented as mean, n=6 mice from each group. Four AKI candidate genes—Tnf, Ngal, Icam-1, and Kim-1—were selected for further in-depth epigenetic analysis.



**Fig.2. Renal cortical Tnf, Ngal, Kim-1 and Icam-1 expression following unilateral kidney I/R and LPS injection**

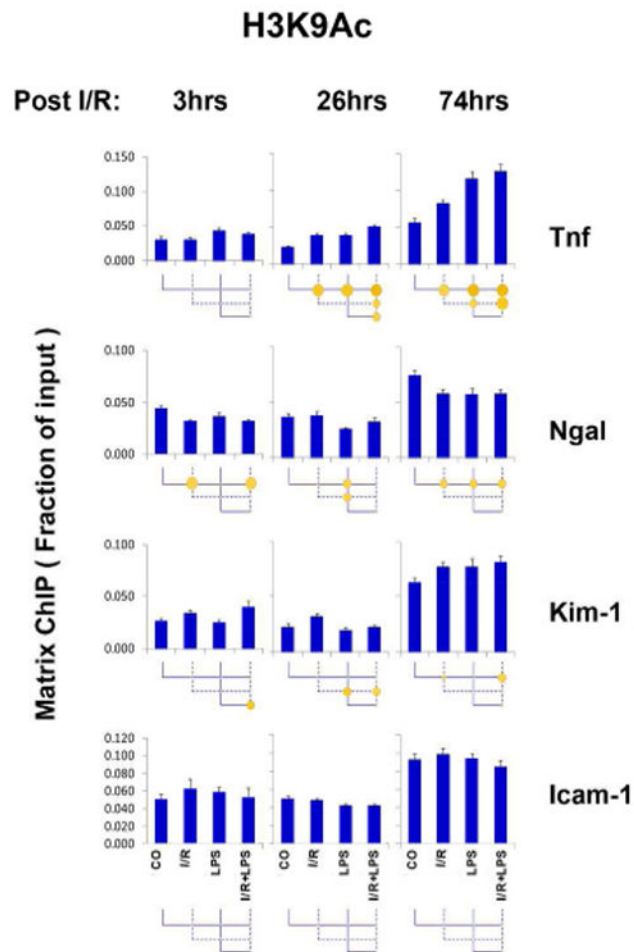
Total RNA from mice renal cortex was used in RT reactions with oligo dT. cDNA was used in real time PCR with gene specific primers (Table S1, supplement). mRNA level of a given gene in each sample was normalized to  $\beta$ -actin transcript. Data are represented as mean  $\pm$  SEM, n= 6 mice in each group. Statistical differences between two means (p value) are shown by the size of the solid circle under the x-axis: p<0.05 by small circle, p<0.01 by large circle, and no circle indicating the differences are not statistically significant (Methods and Fig.S1, supplement).





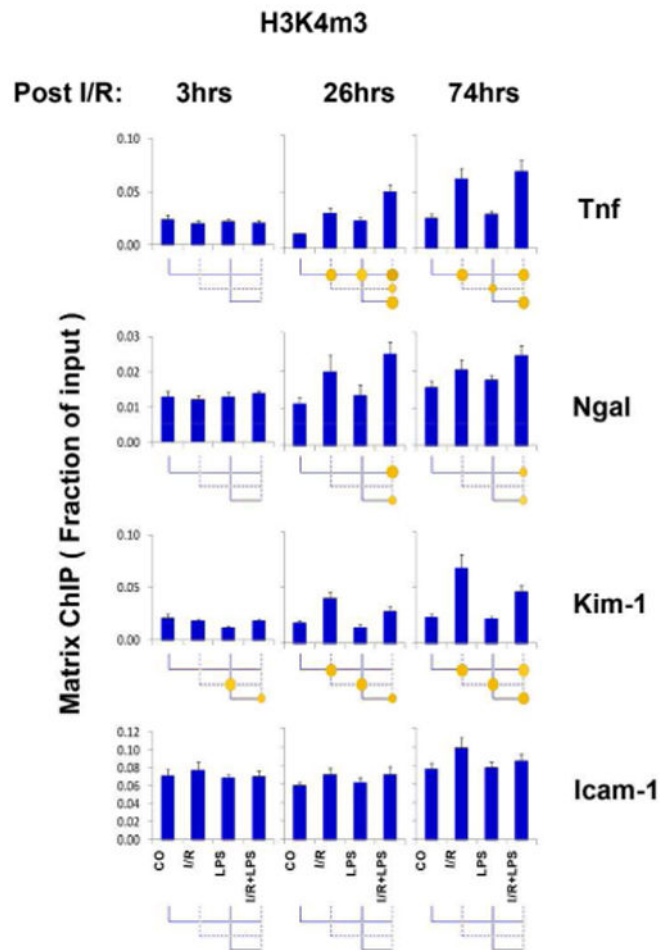
**Fig.3. RNA polymerase II (Pol II) Tnf, Ngal, Kim-1 and Icam-1 genes following unilateral kidney I/R and LPS injection**

Sheared cross-linked renal cortex chromatin from mice were assayed using a monoclonal antibody to (4H8) that detects both unphosphorylated and phosphorylated C-terminal domain repeats YSPTSPS. ChIP DNA were analyzed at Tnf, Ngal, Kim-1 and Icam-1 genes in qPCR. Data represent mean  $\pm$  SEM (6 animals from each group), expressed as fraction of input.



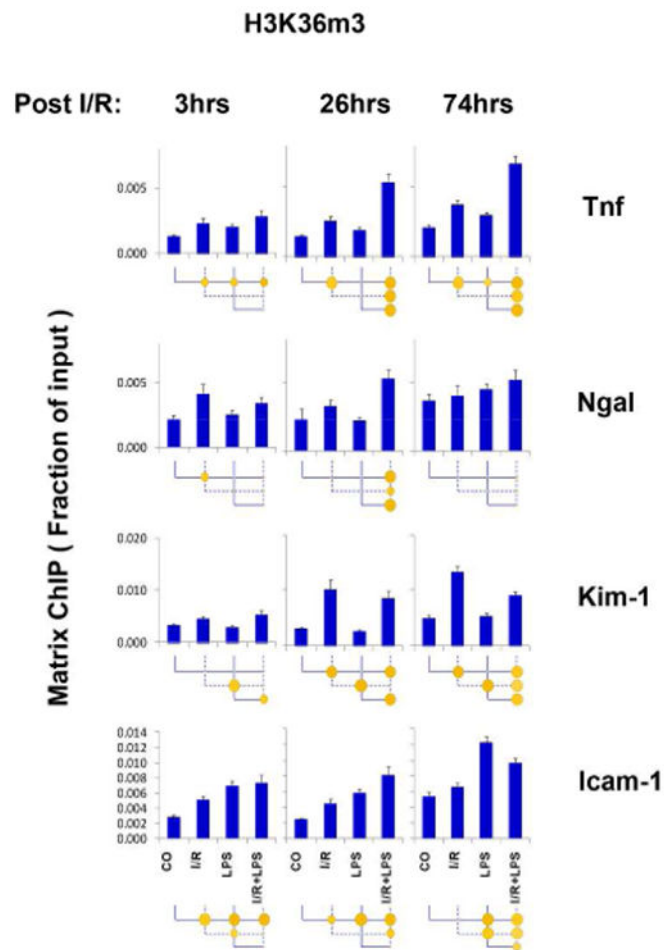
**Fig.4. Permissive histone H3 lysine 9 acetylation (H3K9Ac) at Tnf, Ngal, Kim-1 and Icam-1 genes following unilateral kidney I/R and LPS injection**

Sheared cross-linked renal cortex chromatin from mice were assayed using H3K9Ac antibody. Data represent mean  $\pm$  SEM (6 animals from each group), expressed as fraction of input.

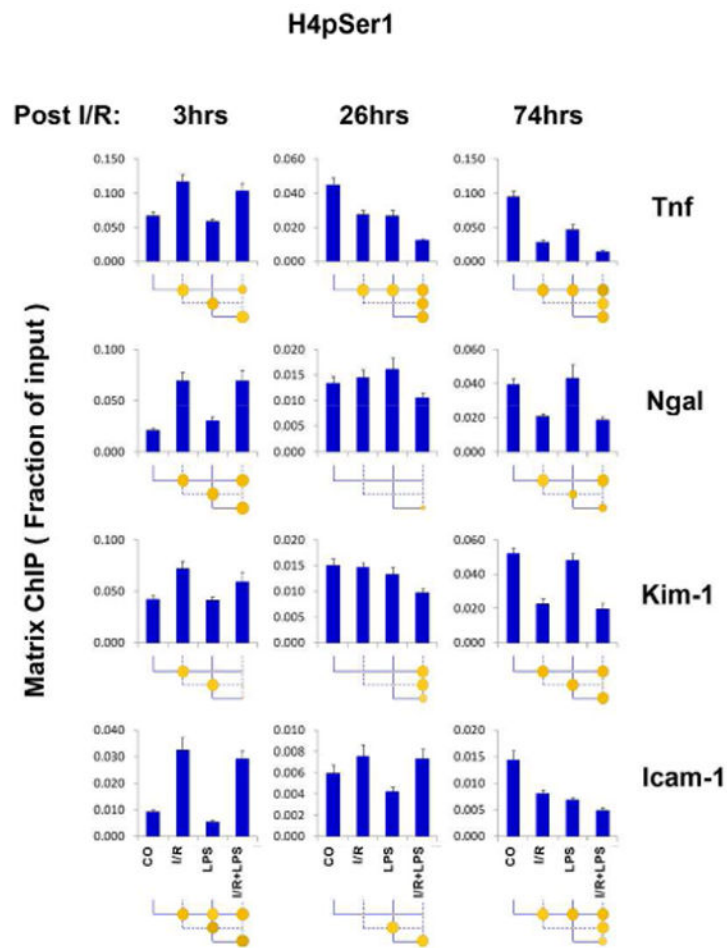


**Fig.5. Permissive histone H3 lysine 4 tri-methylation (H3K4m3) at Tnf, Kim-1, Ngal, and Icam-1 genes following unilateral kidney I/R and LPS injection**

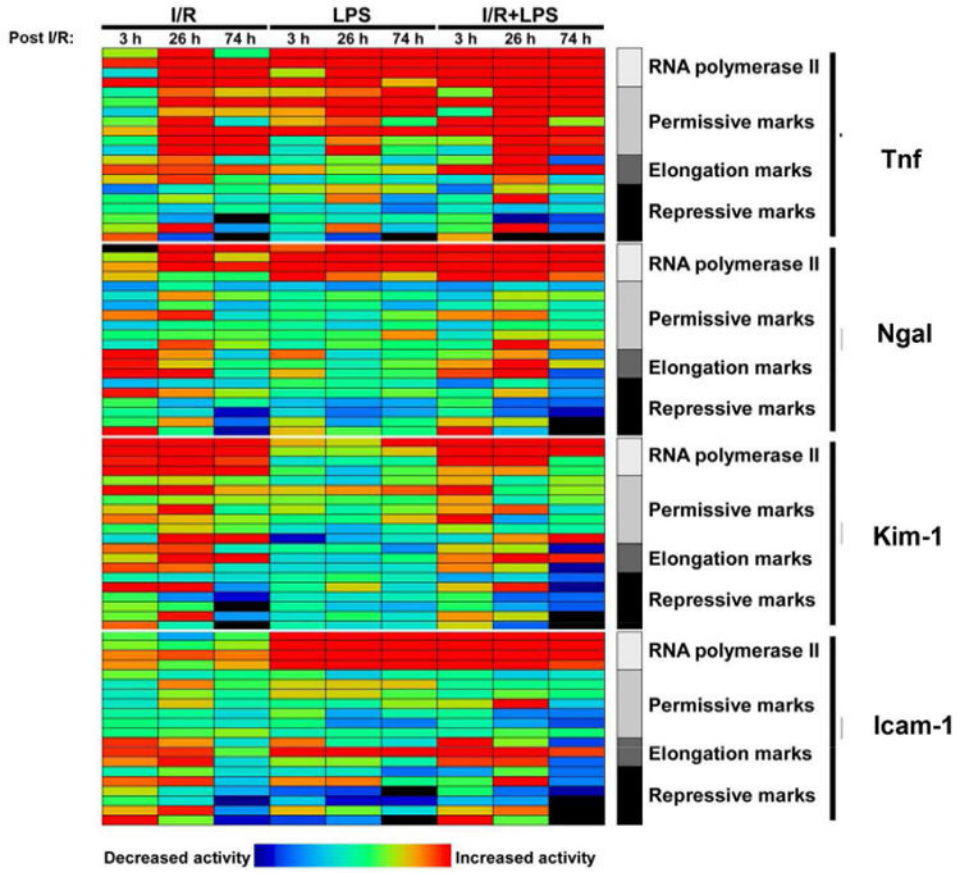
Sheared cross-linked renal cortex chromatin from mice were assayed using H3K4m3 antibody. Data represent mean  $\pm$  SEM (6 animals from each group), expressed as fraction of input.



**Fig.6. Transcription elongation histone H3 lysine 36 tri-methylation (H4K36m3) at Tnf, Kim-1, Ngai, and Icam-1 genes following unilateral kidney I/R and LPS injection**  
 Sheared cross-linked renal cortex chromatin from mice were assayed using H3K36m3 antibody. Data represent mean  $\pm$  SEM (6 animals from each group), expressed as fraction of input.



**Fig.7. Transcription repressive histone H4 serine 1 phosphorylation (H4pSer1) at Tnf, Kim-1, Ngal, and Icam-1 genes following unilateral kidney I/R and LPS injection**  
 Sheared cross-linked renal cortex chromatin from mice were assayed using H4pSer1 antibody. Data represent mean  $\pm$  SEM (6 animals from each group), expressed as fraction of input.



**Fig.8. Integrated transcriptional and epigenetic analysis of Tnf, Kim-1, Ngal, and Icam-1 portraits following unilateral kidney injury**  
 Log-transformed values for mRNA and Pol II levels, as well as several epigenetic modifications during temporal progression of AKI due to I/R, LPS and I/R+LPS are depicted as a heatmap. While there is a general increase in permissive marks and reduction of repressive marks in a time-dependent manner, note the heterogeneous response pattern of individual AKI-induced genes.