TNF drives AKI-to-CKD transition downstream of proximal tubule EGFR

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Abstract:

Inflammation is a key driver of fibrosis and progression of human chronic kidney disease (CKD), often caused or worsened by acute kidney injury (AKI-to-CKD transition). Sustained epidermal-growth-factor-receptor (EGFR) activation in injured proximal-tubule-cells (PTC) is strongly pro-inflammatory and has emerged as a key paradigm in AKI-to-CKD transition and CKD progression. Whether the key Type 1 inflammatory cytokine tumor-necrosis-factor (TNF) has a role in CKD progression and how TNF relates to the PTC-EGFR pathway is unknown, but retrospective analysis of patients using TNF biologic inhibitors suggests that TNF inhibition reduces incident CKD and CKD progression in humans. Here, we compared mice treated with control, TNF inhibition (murine etanercept, soluble TNF-scavenger), EGFR inhibition (erlotinib, EGFR-kinase-inhibitor) or their combination in an AKI-to-CKD bilateral renal-ischemia-reperfusion model. TNF- or EGFR-inhibition did not affect initial kidney injury, but significantly overlapped in reducing kidney injury-upregulated cytokines and equally strongly reduced kidney fibrosis, while combination treatment had no additive effect, suggesting EGFR and TNF act in the same fibrosis pathway. TNF exerted its profibrotic effects downstream of PTC-EGFR, as TNF-inhibition did not affect tubular EGFR activation *in vivo.* Consistent with this, TNF PTC-KO did not reduce inflammation or fibrosis, suggesting that PTC-derived TNF does not contribute to profibrotic PTC-EGFR activation. Kidney single-cell RNAseq analysis identified macrophages, dendritic cells and T cells, but not PTC, as dominant TNF sources after AKI. Only EGFR inhibition, but not TNF inhibition significantly blocked injury-induced kidney ingress of chemokine-receptor-2 (CCR2) positive cells and accumulation of macrophages, however, macrophage numbers where equal one month after AKI independent of treatment. Thus EGFR inhibition reduces ingress and accumulation of TNF-producing proinflammatory and profibrotic immune cells whereas TNF inhibition mechanistically largely acts by neutralizing their proinflammatory and profibrotic activities. Our work provides mechanistic background to motivate examination of TNF pathway inhibition in human AKI or CKD.

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Introduction

Inflammation is a major driver of fibrosis and progression of chronic kidney disease (CKD) resulting in progressive loss of kidney function. Similarly, acute kidney injury (AKI) can lead to persistent pro-fibrotic inflammation (AKI-to-CKD transition), in particular when the insult is severe, repetitive or chronic[1,2]. We showed that injured proximal-tubule-cells (PTC) in mice and humans upregulate a-disintegrin-and-metalloprotease-17 (ADAM17)-dependent release of both, the profibrotic epidermal-growth-factor-receptor (EGFR) ligand amphiregulin (AREG) and of tumor-necrosis-factor (TNF), a key proinflammatory cytokine that orchestrates Type 1 immune responses[3]. ADAM17- or AREG-PTC-knockout (KO) reduced PTC-EGFR activation, immune cell accumulation, inflammation and fibrosis after AKI in mice[4,5], identifying PTC-EGFR activation as a key proinflammatory and profibrotic event in the development of fibrosis after AKI (AKI-to-CKD transition). Similar results were obtained with chemical EGFR inhibition with erlotinib[6]. However, the general role of TNF in AKI or AKI-to-CKD transition remains ill-defined and the role of PTC-TNF is unknown. In particular, how TNF action in the injured kidney relates to activation of the PTC-EGFR pathway *in vivo* is unresolved.

In humans, CKD progression is strongly associated with increased serum levels of TNF and its soluble receptors TNFR1 and TNFR2[7–9], which can act as decoy receptors for TNF, and like TNF are also released by ADAM17. A recent large retrospective propensity-matched cohort study[10] and a smaller study[11] suggested that anti-TNF biologics, such as etanercept (TNFR1-Fc), reduce incidence and progression of CKD in patients with rheumatoid arthritis. In rodents only limited data is available regarding the role of TNF inhibition in kidney injury and profibrotic inflammation. Early kidney injury markers decreased when rats subjected to ischemia-reperfusion injury (IRI) were pre-treated with TNF inhibition by etanercept, but no late fibrosis outcomes were measured[12]. TNF inhibition with soluble pegylated-TNFR1 in rats reduced early fibrosis markers after UUO[13], but another UUO study in TNF-global-KO mice showed increased fibrosis[14]. The dominant cellular sources of TNF in the injured kidney were not determined in these studies. Several studies that reported effects of the human TNF inhibitor infliximab in rodent kidney injury models focusing on early injury[15–18] have been called into question since it was shown that infliximab does not bind to rodent TNF[19], suggesting that observed effects by infliximab were independent of TNF neutralization. Taken together, these findings warrant a reevaluation of the role of TNF inhibition in kidney injury and fibrosis.

In order to study the role of TNF in kidney injury and profibrotic kidney inflammation, we compared mice treated with vehicle (control), murine etanercept (soluble TNF-scavenger), erlotinib (EGFR-kinase-inhibitor) or a combination of both in an AKI-to-CKD bilateral renal-ischemia-reperfusion model. Here, we show that TNF inhibition does not interfere with initial kidney injury and provide evidence that EGFR activation and TNF act in the same fibrosis pathway, as inhibition of either pathway is not additive but

equally protective against the development of fibrosis after AKI. Using proteomic cytokine profiling, bulk mRNAseq, single-cell RNAseq (scRNAseq), *in vitro* studies and the analysis of TNF-PTC-KO mice, we show that the profibrotic actions of injury-induced TNF in the kidney are functionally downstream of PTCs and PTC-EGFR activation, and do not depend on PTC-TNF release, but rather on release of TNF by immune cells, in particular profibrotic macrophages. While TNF inhibition and EGFR inhibition significantly overlapped in their negative effects on kidney-injury induced expression of cytokines, only EGFR kinase inhibition strongly reduced accumulation of inflammatory cells early after kidney injury, while TNF inhibition did not. However, both treatments result in the same reduction of macrophages over the course of disease progression. Thus, mechanistically, TNF acts downstream of PTC-EGFR and the observed benefits of TNF inhibition in PTC-EGFR-driven fibrosis are predominantly due to the capture of TNF released downstream of PTCs by activated profibrotic immune cells, in particular macrophages.

Results

TNF or EGFR inhibition are equally effective in protecting mice from AKI-to-CKD transition

We used an AKI-to-CKD severe bilateral renal ischemia-reperfusion (IRI) model for our studies. In order to conclusively establish that this model leads to significant progressive loss of GFR when observed over a long time frame, we evaluated mice after bilateral IRI or sham operation for a period of 6 months (180 days) (**Figure 1A**). Bilateral IRI caused significant AKI with elevations of serum blood-urea-nitrogen (BUN) and creatinine (determined by mass spectrometry) on Day 1 after AKI. BUN and creatinine returned to baseline after 28 days. In sham mice, only minimal changes of baseline BUN or creatinine were detected (**Supplemental Figure1A+B**). As is frequently observed in patients with AKI and due to renal compensatory mechanisms, glomerular-filtration-rate (GFR), assessed trans-dermally by detecting the excretion of injected FITC-sinistrin[20], returned to normal by 28 days after injury (**Figure 1B**). This occurred despite the fact that at this time point severe fibrosis is observed in the AKI-injured animals as compared to sham-operated animals, as we and others previously documented[4–6,21] and we will also show later in Figure 1G+H. To assess whether established fibrosis at Day 28 would lead to progressive renal failure, we measured GFR over 6 months. All animals showed normal baseline GFR. GFR remained stable in sham-operated animals, but declined progressively in AKI-injured animals starting 3-4 months after injury and leading to a total loss of 40% of baseline GFR by six months after AKI (**Figure 1B**). Serum BUN and creatinine measured over the same time period were normal, highlighting the known insensitivity of these markers for determination of early kidney functional decline. This indicates that our rodent AKI model behaves as predicted from studies in patients with AKI that progress to CKD over time (AKI-to-CKD transition)[1,2].

To assess the effects of TNF inhibition on kidney injury and fibrosis and to compare it to inhibition of profibrotic EGFR activation, our current “gold standard” in mice, we compared bilateral IRI-injured mice either treated with vehicle (control), murine etanercept (ET, dosage 10 mg/kg twice a week, TNF scavenger), erlotinib (ER, dosage 80 mg/kg daily, EGFR kinase inhibitor) or a combination of both drugs (COMB) over a time frame of 28 days (**Figure 1C**). Etanercept was effectively delivered into the interstitial compartment of the kidney, as shown by injection of fluorescently labeled etanercept (**Supplemental Figure 2A**). Labeled etanercept colocalized to some degree with vascular endothelial cell marker CD31, but was also found isolated in the extravascular interstitial space between tubule cells (**Supplemental Figure 2B**). Irrespective of treatment, all injured mice showed the same serum BUN or creatinine at baseline and similarly elevated levels on Day 1 after AKI. Both injury biomarkers recovered to baseline by Day 28 at the same rate in all mice (**Figure 1D+E**). GFR also returned to baseline in all treatment groups by Day 28, as predicted by compensatory mechanisms mentioned above (**Figure 1F**). Together these findings show that TNF or EGFR inhibition and their combination have no significant effect on initial kidney injury and the recovery of biomarkers. As expected, however, vehicle-treated mice showed significant kidney fibrosis as measured by Masson trichrome stain (**Figure 1G**) or Picrosirius red stain (**Figure 1H**). Yet surprisingly, we found that TNF inhibition was as protective against the development of AKI-induced fibrosis as EGFR inhibition, with combination treatment providing no additional benefit (**Figure 1G+H**), suggesting that TNF and EGFR act in the same fibrosis pathway.

EGFR and TNF inhibition strongly overlap in exerting negative effects on cytokine protein expression in the AKI kidney

Based on these results, we hypothesized that TNF inhibition should affect the same or similar proinflammatory and profibrotic molecules in the AKI kidney as does EGFR inhibition, in order to cause similar protection from AKI-induced fibrosis. To gain insights into this, we performed proteomic analysis of cortical kidney lysates of IRI-injured mice treated with vehicle, TNF inhibition, EGFR inhibition, or their combination on Day 2 after injury for the expression of 98 pro-inflammatory/pro-fibrotic cytokines, chemokines and related molecules (in the following simply referred to as cytokines). TNF inhibition (etanercept, ET) indeed overlapped very significantly in the suppression of cytokines with EGFR inhibition or combination (COMB) treatment. Overall, individual treatments had predominantly negative effects on the expression of cytokines, with EGFR inhibition affecting more targets than TNF inhibition or combination treatment (**Figure 2A**). The overlap of TNF inhibition with EGFR inhibition or combination treatment was very significant, with only three targets affected solely by TNF inhibition. This suggests a strong functional overlap of both treatments and also that the overlapping target genes might be particularly important for the observed similar effects of EGFR or TNF inhibition in reducing AKI-to-CKD

transition. Only very few measured cytokines were upregulated as a result of drug treatment (**Supplemental Figure 3**). Gene-ontology (GO) term analysis of all affected cytokines revealed an even more significant overlap between all treatments on the functional level (**Figure 2B**). Top common GO terms for all affected cytokines showed that treatments overlapped most strongly in their negative effects on the immune response, tissue remodeling and inflammatory cell signaling events. Overall, TNF inhibition (ET) had less significant effects as compared to EGFR inhibition (ER) or combination treatment (COMB). Particularly notable differences were detected in the negative effects of treatments on leukocyte migration and leukocyte cell-cell adhesion, with EGFR inhibition (ER) or combination treatment (COMB) significantly more effective than TNF inhibition (ET) (**Figure 2C**). A complete list of affected cytokines and their functional GO terms stratified by treatment condition is shown in **Supplemental Table 1**. All three treatment conditions overlapped in their negative effects on kidney injury-induced expression of six cytokines (**Figure 2D**), suggesting that they might be particularly central to the similar effects of individual treatment conditions on preventing AKI-to-CKD transition: TNF and interferon gamma (IFNG), an early response cytokine that orchestrates activation of the innate immune system were the two top reduced cytokines by all treatments, suggesting TNF and IFNG play an important role in the profibrotic inflammatory response in AKI-CKD-transition. Complement-factor-5 (C5), a strong chemoattractant for immune cells, including neutrophils and monocytes, p-Selectin, a mediator of the adhesion of immune cells such a neutrophils or monocytes to endothelial cells, and the innate immune system activator and regulator of cell migration Serpin E1 (also called plasminogen activator inhibitor 1) represented other key downregulated cytokines, suggesting that their reduction might be central to the reduced inflammatory response and reduced cell migration invoked by all treatments (**Figure 2C**). Interleukin 10 (IL10), a key anti-inflammatory cytokine produced by activated immune cells was moderately reduced by all treatments, likely as a result of dampened inflammation overall, one key stimulus for IL-10 secretion (**Figure 2D**). GO term analysis of the six commonly downregulated cytokines revealed similar shared functional features as the overall GO term analysis of all downregulated cytokines (**compare Figure 2C to 2E**), highlighting again that downregulated cytokines that are overlapping or non-overlapping between treatments share the same or similar functions (**Figure 2B**). A complete list of functional GO terms for common downregulated cytokines is shown in **Supplemental Table 2**. Enrichment analysis of transcription factors known to regulate the expression of any one of all downregulated cytokines revealed that negative regulation of NFκB pathway/complex components, NFκB, RelA and Rel, as well as of one of its key downstream targets, the early response gene Jun, might be central to the shared effectiveness of our treatments in preventing AKI-to-CKD transition (**Figure 2F**). Complete results of this enrichment analysis are shown in **Supplemental Table 3**. Taken together, our cytokine analysis provides overall mechanistic insights relative to the effects of TNF or EGFR inhibition in AKI-to-CKD transition and reveals

TNF as a key cytokine in the development of fibrosis after AKI. TNF is negatively affected in its protein expression in the injured kidney either by EGFR or TNF inhibition, strengthening our hypothesis that TNF and EGFR act in the same pathway.

TNF acts downstream of pro-fibrotic PTC-EGFR activation

To determine whether TNF acted upstream or downstream of pro-fibrotic PTC-EGFR activation, which is most pronounced and easily measured early after injury, we assessed PTC-EGFR phosphorylation at Day 2 after AKI in mice treated with either TNF inhibition, EGFR inhibition or their combination (**Figure 3A**). As already shown in Figure 1, all treatment groups showed the same initial kidney injury as measured by serum BUN (**Figure 3B**). Assessing EGFR phosphorylation with a phospho-specific antibody in cortical lysates highly enriched with PTCs revealed that TNF inhibition (ET) did not significantly affect EGFR phosphorylation in PTCs, whereas EGFR inhibition (ER) or combination (COMB) treatment blocked it completely, as expected (**Figure 3C**). Inhibition of the pro-fibrotic PTC-EGFR pathway would be expected to prevent feed-forward upregulation of the profibrotic EGFR ligand AREG upregulated in injured PTCs[4,5]. Consistent with its lack of effect on PTC-EGFR activation and based on kidney bulk mRNAseq analysis at Day 2, TNF inhibition *in vivo* did not interfere with injury-induced AREG upregulation, or upregulation of two other EGFR ligands previously also associated with kidney fibrosis, transforming-growth-factor-alpha (TGFα)[22] and heparin-binding-EGF (HB-EGF)[23] (**Figure 3D**). Of note, based on our published data, TNFalpha and HB-EGF are pro-fibrotic predominantly by inducing AREG expression and release themselves[5]. We previously observed that soluble TNF can lead to AREG release in cultured PTCs *in vitro* and TNF, next to AREG, is also significantly upregulated in PTCs after AKI[4]. We thus asked whether PTC-TNF has a role in fibrosis *in vivo*. We created PTC-TNF-knockout mice using SLC43A1-Cre as done in previous studies[4,5] and subjected them to the classic unilateral-ureteral-obstruction (UUO) kidney fibrosis model we have used for confirmation of IRI findings in all our relevant studies. PTC-TNF KO mice were not protected against kidney-injury induced fibrosis in the UUO model at Day 7 after uretera; ligation[4,5] as shown by measurements of the fibrosis markers fibronectin, alphaSMA and collagen 1 in kidney tissue (**Figure 3E**), suggesting the PTC-TNF release is not critical for pro-fibrotic PTC-EGFR activation *in vivo* and that the pro-fibrotic actions of TNF do not depend on TNF released from PTCs *in vivo*. Our results thus suggest that TNF inhibition acts downstream of pro-fibrotic AREG-induced PTC-EGFR activation.

EGFR but not TNF inhibition reduces recruitment of immune cells in the injured kidney early after AKI

Using single-cell RNAseq (scRNAseq) of the kidney of sham-operated or IRI-injured mice with AKI, we aimed to determine cellular sources of TNF other than PTCs in the injured kidney that might explain the observed anti-fibrotic effects of TNF inhibition. As expected, in sham operated mice TNF expression was very low across all cell types in the kidney (data not shown). Consistent with the lack of protective effect of PTC-TNF-knockout, scRNAseq at Day 1 after AKI identified only low TNF expression in PTCs. However, high expression of TNF was detected in neutrophils, which are present in the injured kidney only early after injury, in dendritic cells and in macrophages. Most macrophages that accumulate in the kidney after injury are known to be derived from circulating monocytes that express chemokine-receptor-2 (CCR2)[24,25]. Macrophage persistence in the kidney after injury strongly correlates with maladaptive repair and the development of kidney fibrosis[26,27] (**Figure 4A**). Mass cytometry analysis of sham and injured kidney on Day 2 after AKI using the macrophage markers CD64 (classical macrophage marker) and CD80 revealed that only EGFR inhibition, but not TNF inhibition, strongly reduced the accumulation $CD64^+$ macrophages in the kidney; CD80 showed a significant trend to reduction (**Figure 4B**). This was confirmed by bulk mRNAseq analysis of Day 2 AKI kidneys, which showed that only EGFR but not TNF inhibition reduced expression of CD45 (general immune cell marker), CD64 (classical macrophage marker) and F4/80 (macrophage/dendritic cell marker) in the injured kidney. In addition, kidney expression of CCR2 was also only downregulated by EGFR inhibition, but not by TNF inhibition (**Figure 4C**). CCR2 is an immune cell receptor stimulated by monocyte-chemoattractant-protein-1 (MCP-1/CCL2) which acts as a key recruitment signal for circulating monocytic precursors of macrophages to sites of tissue injury. Thus EGFR inhibition blocks recruitment of monocytes and thus accumulation of TNF-producing kidney macrophages, while TNF inhibition does not interfere with macrophage accumulation after injury at early time points. However, at late time points after injury on Day 28, when all treatments studied showed the same beneficial effect in reduction of fibrosis (see Figure 1G+H), F4/80 positive macrophage numbers in kidneys treated with TNF inhibition were similarly low as after EGFR inhibition or combination treatment (**Figure 3D**). Our results thus show that both treatments ultimately prevent the chronic accumulation of profibrotic immune cells in the kidney. EGFR inhibition reduces early recruitment and thus accumulation of TNF producing immune cells in the kidney, in particular macrophages, whereas TNF inhibition blunts the activation and profibrotic activity of these same immune cells and thereby prevents their chronic accumulation and disease progression.

Discussion

Our data reveal that in terms of preventing chronic pro-fibrotic inflammation in AKI-to-CKD transition EGFR and TNF inhibition are equally effective, because TNF acts as a major effector downstream of profibrotic PTC-EGFR signaling. PTC-EGFR inhibition reduces recruitment of profibrotic immune cells early after AKI, limiting profibrotic inflammation in general, whereas TNF inhibition does not act directly on PTC, but rather on TNF produced by profibrotic immune cells that are recruited and accumulate dependent on PTC-EGFR signaling. Consistent with our data, TNF inhibition has also been shown to decrease glomerular inflammation and glomerulosclerosis (glomerular fibrosis) in diabetic nephropathy. In diabetic Akita mice, anti-TNF antibody reduced glomerular macrophage accumulation, glomerulosclerosis, albuminuria and prevented reduction in glomerular filtration rate at 18 weeks of age[28], consistent with an anti-fibrotic effect of TNF inhibition in inflamed glomeruli. This study also highlighted the importance of TNF expression in macrophages for the induction of fibrosis in the injured kidney, as suggested by our findings in mice treated with TNF inhibition and our scRNAseq data of the AKI kidney. In the streptozosin (STZ) diabetic model, TNF-macrophage-KO with CD11b-Cre reduced BUN and creatinine elevations, glomerular macrophage accumulation, albuminuria and TNF mRNA and protein in the kidney at 12 weeks after STZ[28]. Our scRNAseq data of the AKI kidney also suggested that dendritic cells are possible important sources of profibrotic TNF. Consistent with this, Dong et al. showed that CD11c positive dendritic cells represent a major source of TNF early after renal IRI (24hours)[29]. The role of dendritic cells in kidney fibrosis has not been studied to our knowledge, however, dendritic cells and dendritic cell-derived TNF have been associated with fibrosis in the liver[30]; in addition, dendritic cells are possibly also involved in lung fibrosis[31,32].

Complicating any analysis of TNF targeting agents is the fact that beyond soluble TNF they can also bind cellular uncleaved transmembrane TNF, which preferentially binds to TNFR2, unlike soluble TNF, which preferentially binds to TNFR1. TNFR2 is expressed in immune cells and requires cell-cell contact for activation, with the ligand and receptor being expressed in opposing cells[33]. While TNFR1 activation mediates the classic proinflammatory injurious actions of soluble TNF, TNFR2 activation by transmembrane TNF can have either proinflammatory or anti-inflammatory/pro-repair effects, depending on the cellular context[34]. Etanercept could thus interfere with via TNFR2, possibly explaining some of our results. For the same reason results in TNF global knockout mice have to be interpreted carefully, because they are missing soluble and transmembrane TNF. This possibly explains why some of the earlier studies of TNF inhibition[13] or of TNF global knockout animals[14] in the UUO model yielded divergent results. In contrast to the study using UUO and soluble pegylated-TNFR1 in rats[13], etanercept did not reduce early kidney injury, which could have (but does not) explain our observed reduction in fibrosis.

In previous work, we noted that soluble TNF can induce moderate ADAM17-mediated release of AREG in proximal tubule cells *in vitro*[4], allowing the possibility that PTC-TNF *in vivo* might act upstream of profibrotic PTC-EGFR signaling and enhance sustained AREG-induced profibrotic PTC-EGFR activation[5]. However, the lack of effect of TNF inhibition on PTC-EGFR activation or on the injury-induced upregulation of AREG shown in our current study strongly suggests that TNF-induced AREG release in PTCs as observed *in vitro* does not significantly contribute to the development of AKI-induced fibrosis *in vivo.* Our studies in TNF-PTC-KO mice, which we show are not protected from injury-induced fibrosis, further validate this point and exclude a significant role of PTC-TNF in fibrosis. This conclusion is consistent with our previous observations that injection of sAREG alone into injured ADAM17-PTC-KO mice which are unable to release PTC-AREG or PTC-TNF[4], sufficed to restore inflammation, macrophage ingress and fibrosis [5]and that AREG-PTC-KO phenocopies ADAM17-PTC-KO in protection from AKI-to-CKD transition[5].

Although AKI-to-CKD transition with progressive loss of GFR is by now a well-established clinical concept[1,2,35–37], its dynamic development has to our knowledge not been shown on the GFR level in a mouse model. Using the same bilateral IRI AKI model, others confirmed our findings on early injury resolution with normalization of biomarkers over 14 days, but found that Creatinine was still normal at 6 months and only elevated by 12 months after AKI[38] revealing the higher sensitivity of GFR measurements in our studies, which showed decrease in GFR starting at 3-4 months after AKI that was not detectable by serum BUN or Creatinine measurements.

Our studies raise the possibility that TNF or EGFR inhibition could be used clinically in kidney disease. EGFR inhibition has largely been used in the treatment of cancer. Because EGFR signaling is important for homeostasis, proliferation and wound healing in many epithelial cells, EGFR inhibition typically leads to diarrhea and various cutaneous side effects that are in part cosmetically relevant and disfiguring, but can also lead to wound healing defects and skin infections. Blunting of epithelial repair by EGFR inhibition leading to delayed renal recovery after AKI has been observed in mice[39]. For these reasons, EGFR inhibition would likely not represent a viable therapeutic strategy clinically. This may change in the future, as a recent study showed that fibroblast-growth-factor-7 (FGF7) can rescue skin barrier defects in the context of absent EGFR activity[40]. FGF7 or therapeutic modulation of its pathway could potentially be used topically on the skin or orally. TNF inhibition on the other hand has been used in various autoimmune diseases for long periods of time with significant benefits that are weighed against potentially serious adverse effects and risk of morbidity/mortality due to disease progression. Typical adverse effects include injection site reactions, infusion reactions (both usually manageable), neutropenia (usually mild), skin lesions and infections (bacterial, zoster, tuberculosis, opportunistic). Risk of demyelinating disease has been suggested but is not proven. The risk of heart failure remains unclear, as combined analysis of the

two performed relevant trials showed no effect on death or new heart failure, with only few patients possibly experiencing heart failure exacerbations[41]. The risk of malignancy due to TNF inhibition is low but very difficult to separate from increased risk secondary to the relevant autoimmune disease itself or from concomitant treatments, such as methotrexate. Several described autoimmune diseases that can develop in response to TNF inhibitors are rather rare, including reports of such diseases affecting the kidney itself[42–44]. In our view, the opportunity for TNF inhibition or EGFR inhibition in kidney patients could center on short- or medium-term treatment (weeks to 1-2 months, as opposed to long-term treatment in cancer or autoimmune diseases) used relatively early after onset of AKI when AKI-to-CKD transition could be prevented or blunted by anti-inflammatory therapy. TNF inhibition would likely be preferrable to EGFR inhibition based on potential adverse effects on tubular epithelial biology during early repair. Given the high yearly mortality of CKD patients this could be a justifiable approach and be performed at reasonable risk.

Figure Legends

Figure 1: EGFR inhibition, TNF inhibition or their combination are equally effective in reducing AKI-to-CKD transition.

A. Experimental scheme of AKI-to-CKD model. **B.** Monthly glomerular filtration rate (GFR) measurements in Sham and AKI mice over 6 months. GFR decreases starting at 3-4 months after AKI. **C.** Experimental scheme of mice treated with vehicle (V/V), EGFR (ER, erlotinib) or TNF inhibition (ET, etanercept) or their combination (COMB) after AKI. **D.** Serum BUN values. BUN reaches baseline independent of treatment by Day 14 after AKI. **E.** Serum creatinine values on Day 1 and Day 28 after AKI. No difference between treatments in Creatinine elevation after AKI on Day 1 or recovery to baseline at Day 28. **F.** GFR measurements. No difference between treatments at Day 28 after AKI. **G.** Immunofluorescence staining of cortical area at day 28 after AKI. Upregulation of fibrosis markers Fibronectin (green) and αSMA (red) is significantly reduced by all treatments as compared to vehicle treated cells. **H.** Picrosirius red staining of cortical area at day 28 after AKI. Fibrosis is significantly reduced by all treatments as compared to vehicle treated cells. n = 6-10 mice/group. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

Figure 2: EGFR and TNF inhibition have strongly overlapping negative effects on kidney cytokine protein expression after AKI.

A. Cytokines downregulated by each treatment (Mouse XL Cytokine Array Kit, R&D systems) as compared to vehicle treated AKI animals. Etanercept (ET) shows significant overlap with erlotinib (ER) and combination (COMB) (n=3). **B.** Metascape GO-term analysis of all downregulated cytokines. Outer circle denotes treatments; inner circle represents the downregulated cytokines: those which are common between treatments are shown in dark orange and those unique to a particular treatment are shown in light orange color. Purple curves connect identical cytokines and blue curves connect cytokines that belong to the same enriched GO term. Functional overlap between treatments is more significant than protein analysis suggests. **C.** Top common GO terms of all downregulated cytokines. Negative effects on inflammation and leukocyte migration represent top GO terms for ER and COMB and to a lesser but still significant degree for ET. **D.** Downregulated cytokines common to all treatments. TNF and INFG, which drive type 1 inflammation and activation of the innate immune system represent top hits negatively affected by all treatments. **E.** Top common GO terms of common downregulated cytokines. Negative effects on inflammation and leukocyte migration represent top GO terms. **F.** Enrichment analysis of transcription factors known to regulate any of all downregulated cytokines. Regulation of NFκB complex

proteins (NFκB, RelA, Rel) and Jun could be a shared mechanism mediating the antifibrotic effects of all treatments.

Figure 3: TNF inhibition does not affect EGFR activation after AKI *in vivo* and PTC-derived TNF does not contribute to injury-induced fibrosis.

A. Experimental scheme of 2-day inhibitor treatment after AKI. **B.** Serum BUN values Day 1 after AKI in vehicle (V/V), etanercept (ET), erlotinib (ER) or combination (COMB) treated AKI animals (n=5). **C.** Western blot of cortical PTC-enriched kidney lysates using anti-phospho-EGFR (Y1068) antibody. ER and COMB block PTC-EGFR phosphorylation as expected, but ET does not affect PTC-EGFR phosphorylation after AKI (n=4). **D.** Bulk mRNAseq analysis at Day 2. ET does not affect expression of the profibrotic EGFR ligands amphiregulin (AREG), Heparin binding EGF (HB-EGF) or transforming-growth-factor-alpha (TGFA) (n=3). **E.** PTC-TNF knockout (PTC-TNF-KO) compared to littermate *wt* control does not interfere with fibrosis development after AKI as measured by immunofluorescent staining for the fibrosis markers fibronectin, αSMA or collagen 1 (n=3). *$P < 0.05$; **$P < 0.01$

Figure 4: EGFR but not TNF inhibition reduces recruitment of immune cells in the injured kidney early after AKI

A. scRNAseq analysis of AKI kidney Day 1 after AKI compared to sham kidney. AKI upregulates TNF strongly in neutrophils, macrophages and dendritic cells. **B.** Mass cytometry analysis of kidney Day 2 after AKI in vehicle (V/V), etanercept (ET), erlotinib (ER) or combination (COMB) treated AKI animals. ER but not ET significantly reduces the number of CD64 positive macrophages after AKI. A similar trend is seen for the CD80 macrophage marker (n=3). **C.** Bulk mRNA sequencing analysis kidney Day 2 after AKI. Expression of the general immune cell marker CD45, of the macrophage markers CD64, of the macrophage/dendritic cell marker F4/80, and of CCR2, an important immune cell recruitment signal are all significantly reduced by ER but not by ET treatment, suggesting ER but not ET drives recruitment and accumulation of immune cells in the injured kidney (n=3). **D.** Macrophage/dendritic cell immunofluorescent stain with anti-F4/80 (green) Day 28 after AKI in vehicle (V/V), etanercept (ET), erlotinib (ER) or combination (COMB) treated AKI animals. All treatments reduce macrophage and dendritic cell numbers in the AKI kidney by Day 28 (n=6-10). *$P < 0.05$; **$P < 0.01$

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Methods

Animal studies

For all studies adult (8-12 week-old) male mice were used in accordance with the animal care and use protocol approved by the Institutional Animal Care and Use Committee of Washington University School of Medicine, in adherence to standards set in the *Guide for the Care and Use of Laboratory Animals* (8th edition, The National Academies Press, revised 2011).

The TNF floxed mice were a gift from Dr. Nedospasov (Engelhardt Institute of Molecular Biology, Moscow, Russia) and Dr. Grivennikov (Fox Chase Cancer Center, Philadelphia, PA)[45]. The Slc34a1-CreGSE mice were provided by Dr. Humphreys (Washington University, St Louis, MO). For induction of the Slc34a1-driven Cre, mice were injected with 3 doses of tamoxifen 3 mg (every other day) and we allowed 1 week for tamoxifen washout before performing any procedure.

Ischemia for 21 min at 37°C was induced in both kidneys using the flank approach as previously reported[46]. Sham operations were performed with exposure of both kidneys, but without induction of ischemia. Unilateral ureteral obstruction (UUO) was executed as described previously[46]. Sham-operated mice underwent the same surgical procedure except for the ureter ligation.

Animal treatments

Erlotinib hydrochloride was purchased from LC Laboratories (Woburn, MA, USA). It was dissolved in a vehicle consisting of 0.5% methyl cellulose + 1% tween 80 in water at a concentration of 8 mg/ml and stored in aliquots at -20 °C. Erlotinib was administered to animals at a dose of 80mg/kg/day via gavage[6]. Murine etanercept was kindly provided by Pfizer at a concentration of 12.5 mg/ml, it was diluted using PBS and injected to animals at a dose of 10 mg/kg i.p. twice per week. Mice were randomly assigned into five groups and received treatments for 2 or 28 days (starting from the surgery day until day 1 or day 27 respectively) as follows; group 1 (Sham): received both Erlotinib via gavage daily and Etanercept i.p. twice per week, group 2 (V/V): received the vehicle used for dissolving Erlotinib via gavage daily and was injected vehicle PBS i.p. twice per week, group 3 (ET): received Etanercept i.p. twice per week and vehicle used for dissolving Erlotinib via gavage daily, group 4 (ER): received Erlotinib gavage daily and PBS i.p. twice per week, group 5 (COMB): received both Erlotinib gavage daily and Etanercept i.p. twice per week. Mice were sacrificed on day 2 or day 28.

A group of mice received fluorescently labeled etanercept (the VRDye™ 549 kit from LI-COR was used for labeling according to manufacturer's instructions) to confirm delivery of the drug in the kidney interstitium.

Renal function and histology

Serum creatinine was assessed by an LC-mass spectrometry-based assay at the O'Brien Core Center for Acute Kidney Injury Research (University of Alabama School of Medicine, Birmingham, Alabama, USA). BUN levels were measured using the DiUR100 kit (Thermo Scientific) according to the manufacturer's instructions.

Kidney histology was examined in formalin-fixed sections. For each staining, 10 images per kidney throughout the tissue were collected for blinded quantification. Fibrosis severity was quantified in kidney cortex by measuring the Picrosirius red-stained area using ImageJ software[47].

GFR measurements

GFR was measured by monitoring the clearance of FITC-sinistrin (Fresenius-Kabi, Linz, Austria) using the MediBeacon Transdermal GFR Monitor (MediBeacon GmbH, Mannheim, Germany). Briefly, the Transdermal GFR Monitor was attached to the shaved area on the mouse back for 2 min then FITC-sinisterin was injected to mice at a dose of 75 mg/Kg intravenously via the reto-orbital sinus. The fluorometer was allowed to record the change in FITC-sinisterin fluorescence over 1 hr. The device was then removed and connected to PC to download the recorded measurements. GFR was calculated using one compartmental kinetics fitting allowing direct conversion from the FITC-sinisterin elimination half-life obtained from the exponential excretion phase of the curve using a conversion factor.

GFR (μL/min/25gBW) = 3654.2/ $t_{1/2}$ (FITC-sinistrin) (min)

Bulk RNA sequencing

Total RNA was isolated from mouse kidneys using the TRIzol reagent (Invitrogen) following the manufacturers' instructions. Total kidney RNA samples were sequenced by Novogene Corporation Inc and the results were analyzed using GraphPad Prism, version 9.0 (GraphPad Software Inc.).

Protein sample preparation and western blot

Whole kidney lysates were prepared as previously described[4] and analyzed by standard western blot techniques. The primary antibodies used were from Cell Signaling Technology (p-EGFR Y1068 #2234) or from Abcam (GAPDH #ab181602).

Cytokine profile assay

The proteome profiler array was performed using the Mouse XL Cytokine Array Kit (#ARY028, R&D systems, Minneapolis, USA) following the manufacturer's instructions. Briefly, kidneys were homogenized in PBS to which protease inhibitors and 1 % Triton-X were added. Assay membranes were incubated with blocking buffer for 1 hr at room temperature and then with the lysates overnight at 4 ^{0}C. After washing, membranes were incubated with the detection antibody cocktail for 1 hr at room temperature. After another wash, membranes were incubated with Streptavidin-HRP for 30 min at room temperature, washed again and exposed to the Chemi reagent mix. Images were captured using the ChemiDoc Imager (BioRad) and analyzed using the ImageLab software (BioRad). The pixel density of each spot was determined using the reference spots and signal density was compared between different samples to determine the relative changes in the analyte levels between the different samples. Only cytokines reduced or increased by at least 30% with respect to vehicle were considered as downregulated or upregulated respectively. GO enrichment analyses for Biological Processes and transcriptional regulatory networks (TRRUST) were performed using Metascape[48]. Venn diagrams were generated using BioVenn[49]. Heatmaps were produced using GraphPad Prism, version 9.0 (GraphPad Software Inc.).

Immunofluorescence staining

Immunofluorescence staining of the kidney was performed on frozen sections following standard protocols. In brief, cryo-sections of tissue in OCT were washed with PBST (0.05% (v/v) Tween-20 in PBS) for 5 min at room temperature and permeabilized with 1% SDS in PBS for 5 min, followed by three 5 min washes with PBST. After 30 min incubation in blocking buffer (5% BSA and 5% goat serum in PBS), sections were incubated with primary antibodies overnight at 4oC, followed by three 5 min washes

with PBST, 1 h incubation with secondary antibodies (where appropriate), three 5 min washes with PBST and mounting in DAPI-containing medium (Vector Laboratories). The primary Abs included: αSMA (FITC-conjugated, Sigma-Aldrich, #F3777), fibronectin (Abcam, #ab2413), F4/80 (Abcam, #ab6640) and Collagen I (Abcam, # ab34710). For each kidney staining, 10 images per kidney throughout the tissue were collected for blinded quantification. Images were analyzed using the ImageJ software.

Single cell preparation for RNA sequencing and mass cytometry

Kidneys were minced into small pieces (<1mm^3), and incubated in tissue dissociation buffer (1 mg/ml Liberase TM, 0.7 mg/ml Hyaluronidase, 80U/ml DNAse in PBS) for 30 min in 37°C. Single cells were released from the digested tissue by pipetting 10 times and the cell suspension was filtered through a 70 *μ*m sieve (Falcon). 10% Fetal Bovine Serum (FBS) was added to stop the enzymatic reaction. Cells were collected by centrifugation (300 g at 4°C for 5 min) and resuspended in red blood cell lysis buffer (155mM NH4Cl, 10mM KHCO3, 1mM EDTA, pH 7.3) for 1 min at room temperature. After washing in PBS, cells were either used fresh for analysis by scRNAseq or were cryopreserved in liquid nitrogen vapor in freezing media (50% fetal bovine serum, 40% RPMI 1640, 10% DMSO) until analysis by mass cytometry.

Single Cell RNA Sequencing

Single cell RNA sequencing (scRNAseq) analysis of 4 pooled sham or 4 pooled AKI samples was performed as described previously[50]. Briefly, cells were stained with propidium iodide (PI) and live cells were sorted using FACS AriaIII (BD Biosciences). Libraries were prepared utilizing the v2 Chromium™ Single Cell 5′ Library Kit and Chromium instrument (10x Genomics, Pleasanton, CA). Full-length cDNA was amplified and libraries were submitted to Genome Technology Access Center (GTAC) of Washington University in St. Louis for sequencing.

Seurat (version 3.0) was used for clustering, differentiation analysis of gene expression, and data visualization (UMAP, dot plots) using the R software package. Cells with more than 10% mitochondrial genes and < 200 genes or > 3200 genes are excluded. SCTransform was used for normalization, scaling and variance stabilization (https://github.com/ChristophH/sctransform). The scaled values were then subject to principal components analysis (PCA) for linear dimensionality reduction. Markers for cell clusters were identified using the *FindAllMarkers* function in *Seurat*, and cell types were annotated using canonical markers.

Mass cytometry

Single cell preparations were analyzed by mass cytometry as previously described[51]. Briefly, cells were labeled using a previously validated and titrated antibody cocktail for surface markers[51] (all antibodies conjugated by the manufacturer-Fluidigm) diluted in Fluidigm MaxPar Cell Staining Buffer (CSB) (1 hour at 4 ºC). After two washes in CSB, cells were fixed in 2% PFA for 20 min at room temperature, washed, stained with MaxPar Intercalator-IR (Fluidigm) and filtered into cell strainer cap tubes. Data was then acquired on a CyTOF2/Helios instrument (Fluidigm) and analyzed using the CytoBank software.

Statistics

All results are reported as the mean ± SEM. Comparison of 2 groups was performed using an unpaired, 2-tailed t-test or a Pearson correlation analysis where appropriate. Comparison of 3 groups was performed via ANOVA and Tukey's post hoc test. Statistical analyses were performed using GraphPad Prism, version 9.0 (GraphPad Software Inc.). A *P* value of less than 0.05 was considered significant.

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Figure 1: EGFR inhibition, TNF inhibition or their combination are equally effective in reducing AKI-to-CKD transition.

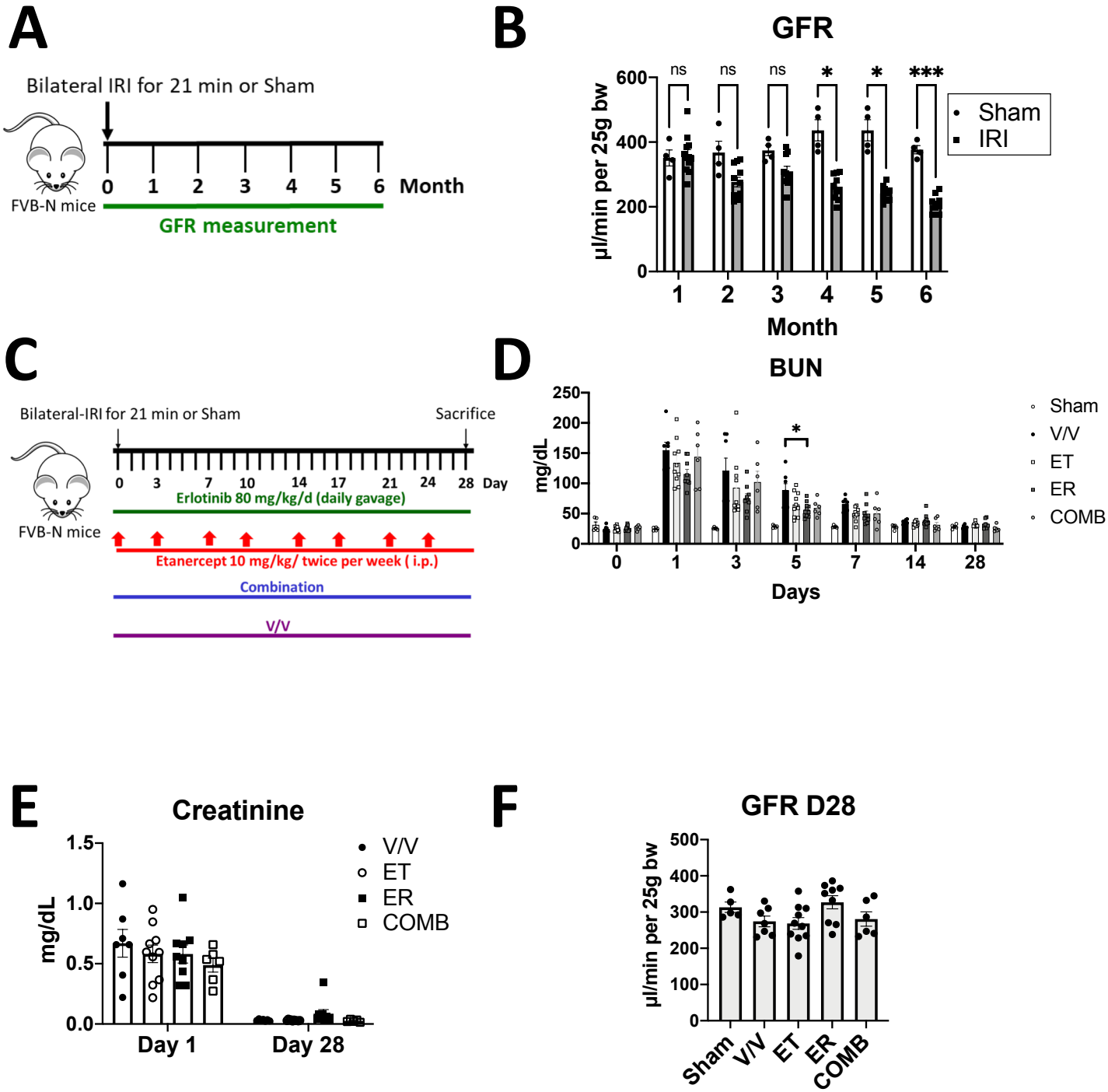
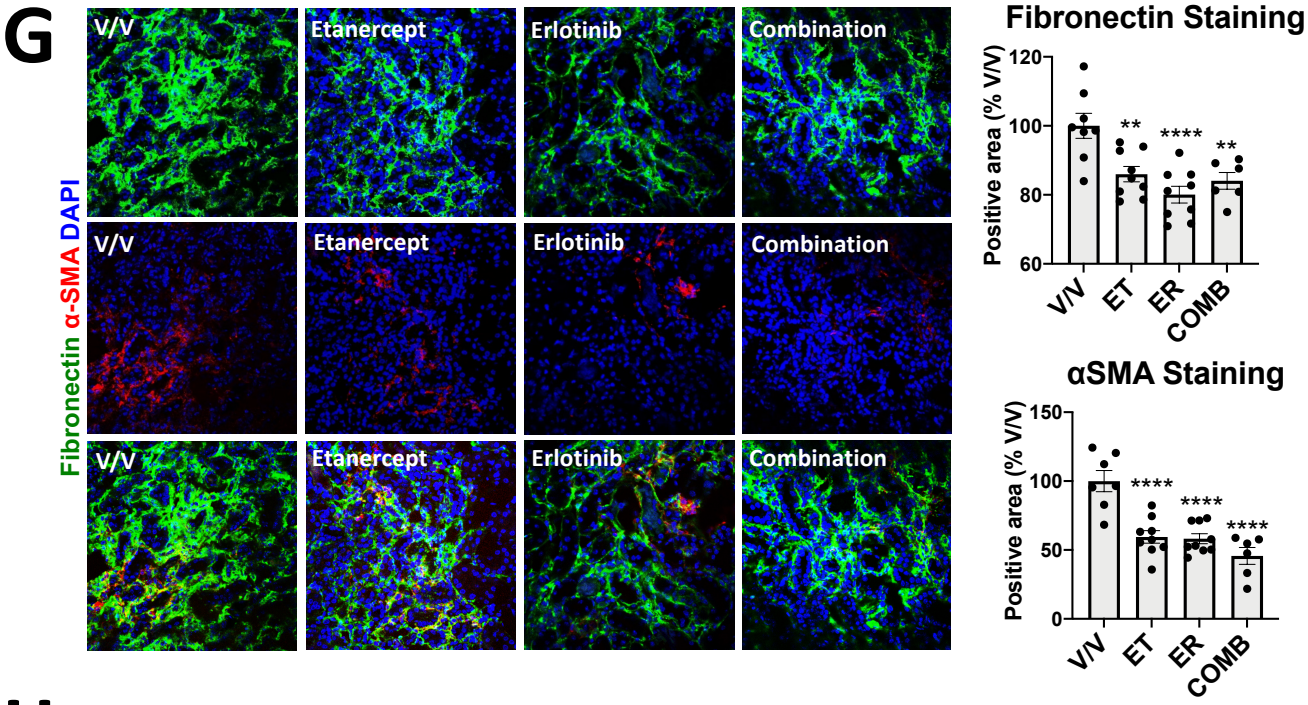


Figure 1 (cont.):



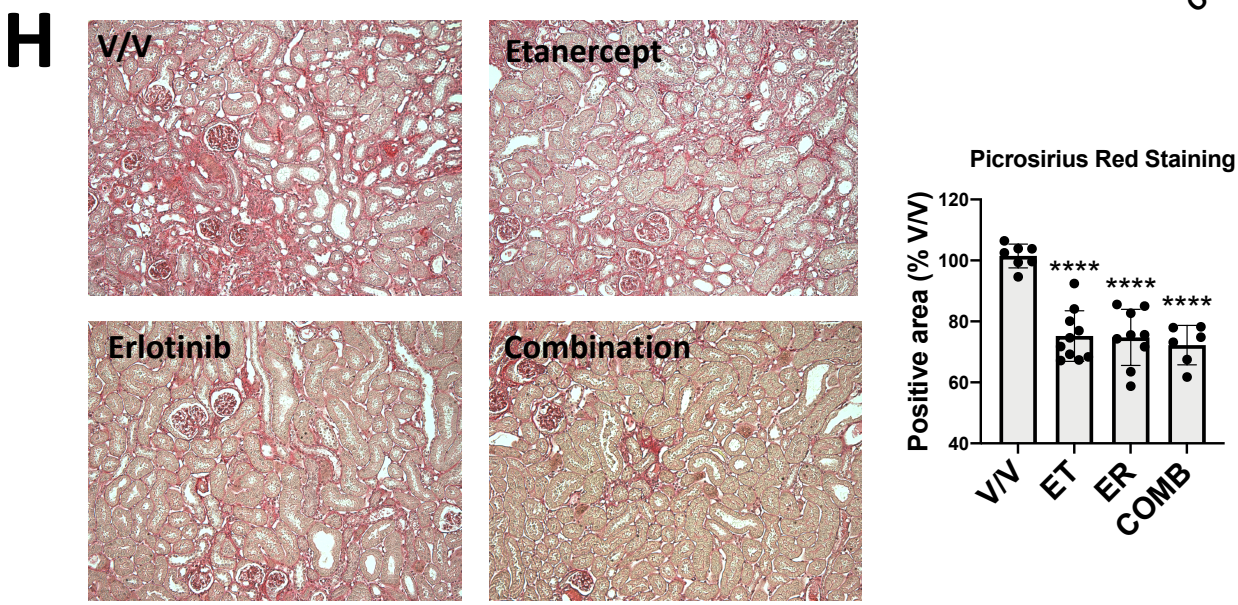
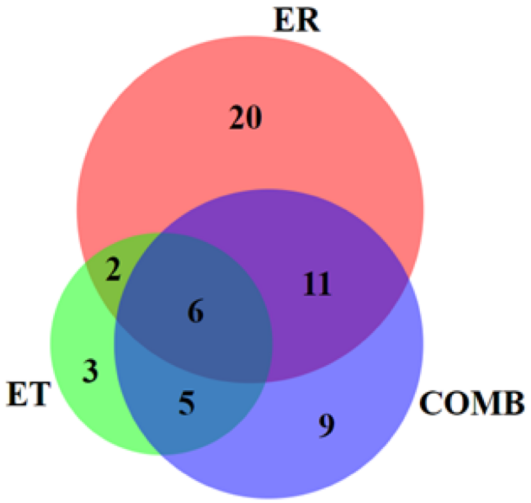
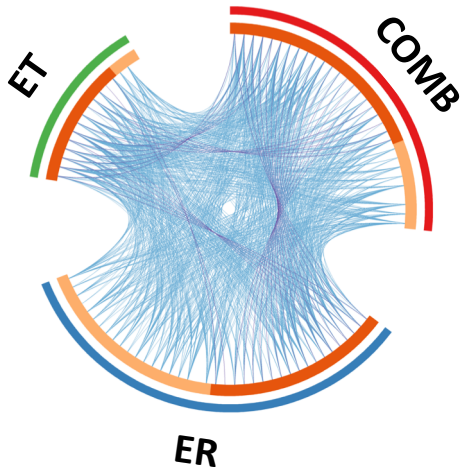


Figure 2: EGFR and TNF inhibition have strongly overlapping negative effects on kidney cytokine protein expression after AKI.

A Down regulated cytokines Kidney Day 2



B Go terms Metascape



C Top common GO terms of all downregulated cytokines

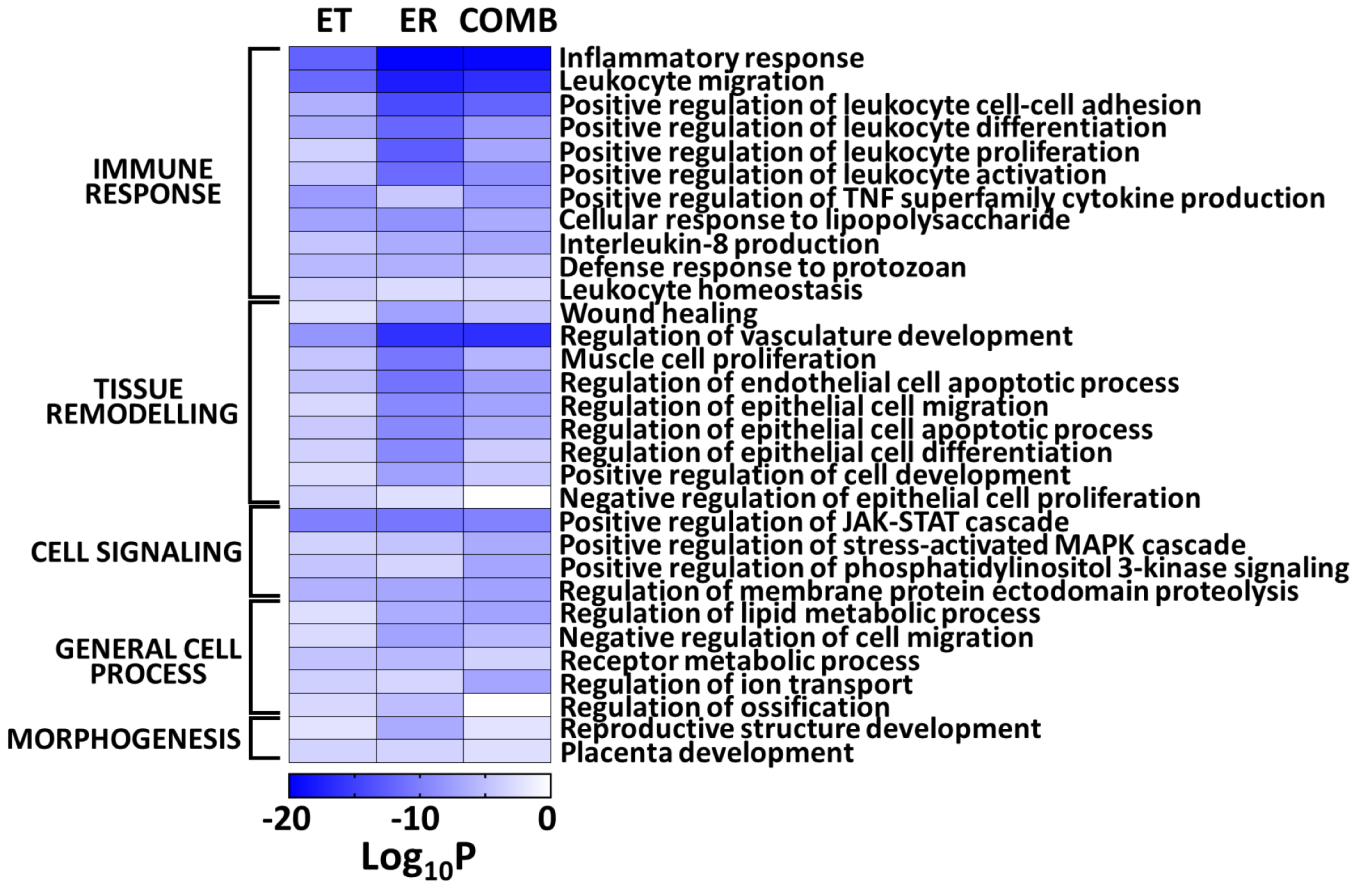
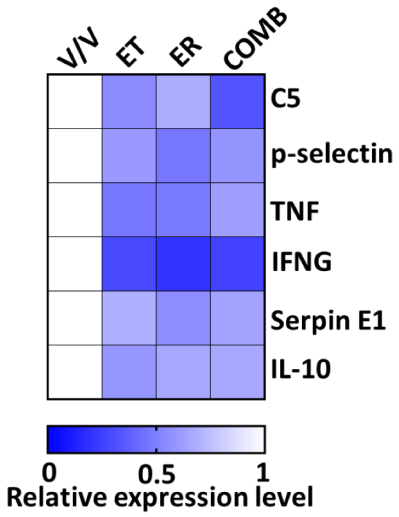
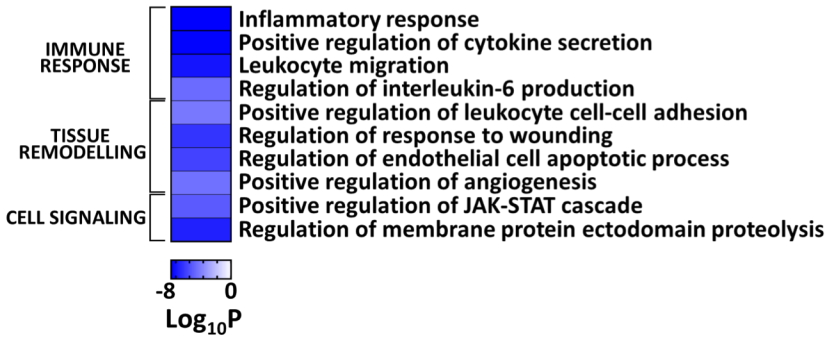


Figure 2 (cont): EGFR and TNF inhibition have strongly overlapping negative effects on kidney cytokine protein expression after AKI.

D Downregulated cytokines common to all treatments



E GO terms: Downregulated cytokines common to all treatments



F Regulated transcription factors for all downregulated cytokines

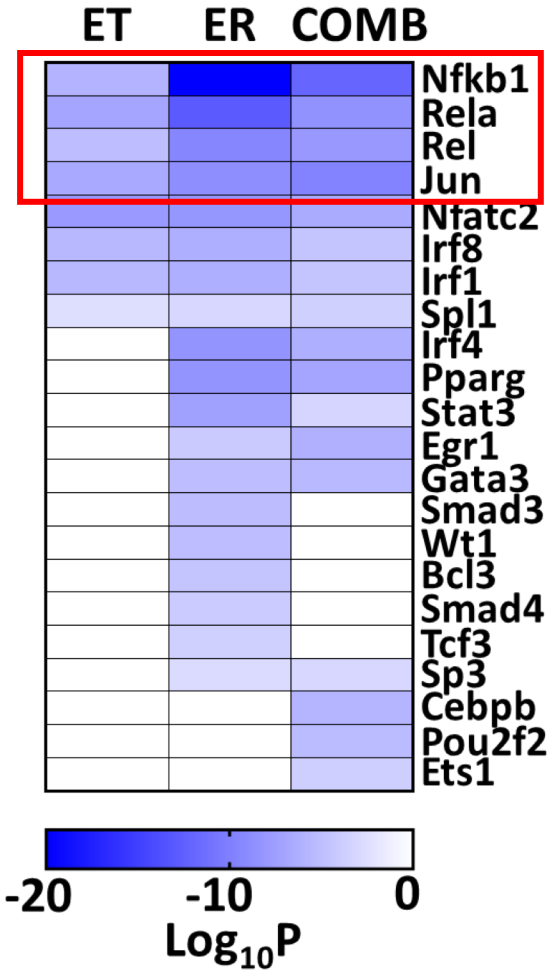


Figure 3: TNF inhibition does not affect EGFR activation after AKI *in vivo* and PTC-derived TNF does not contribute to injury-induced fibrosis.

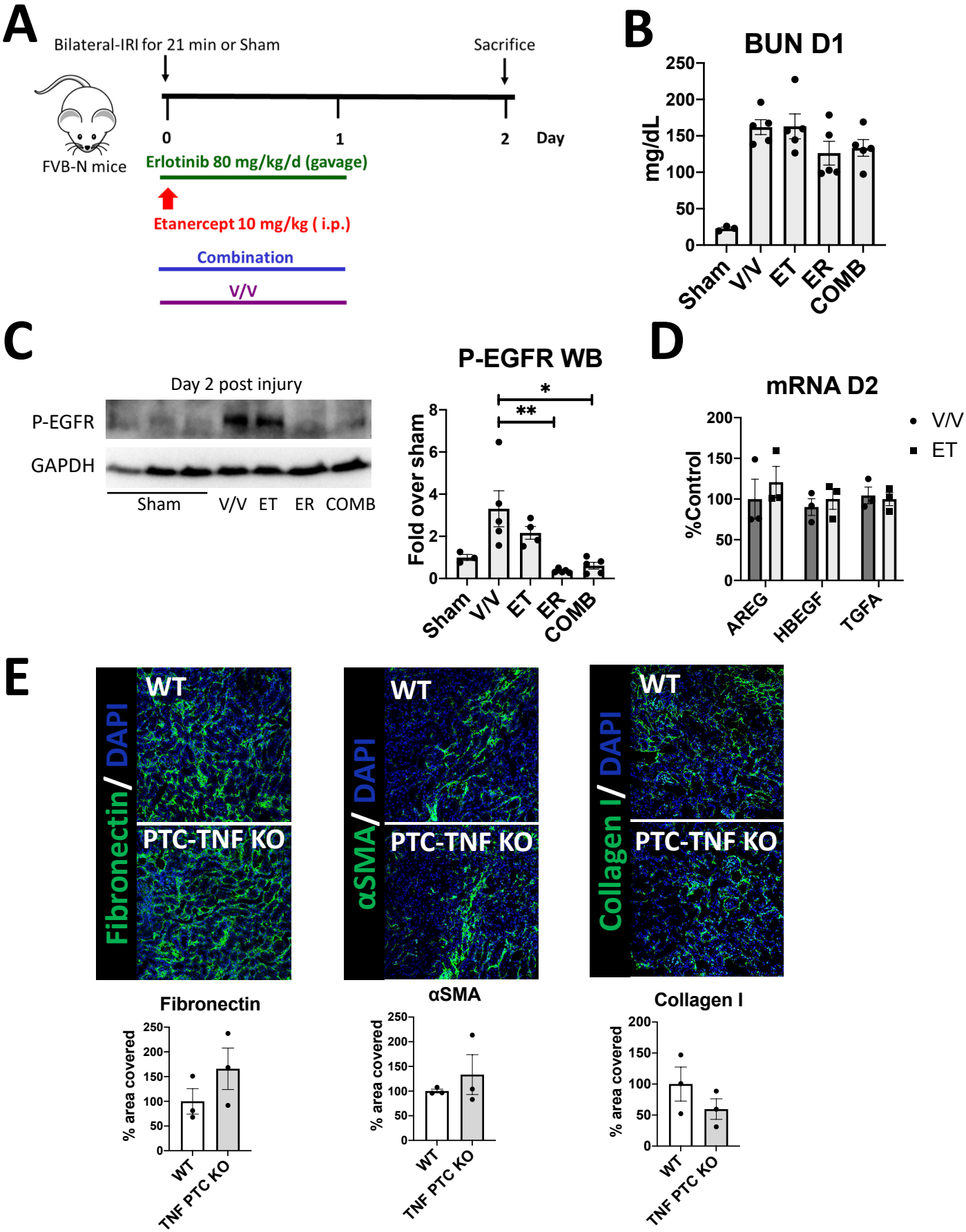
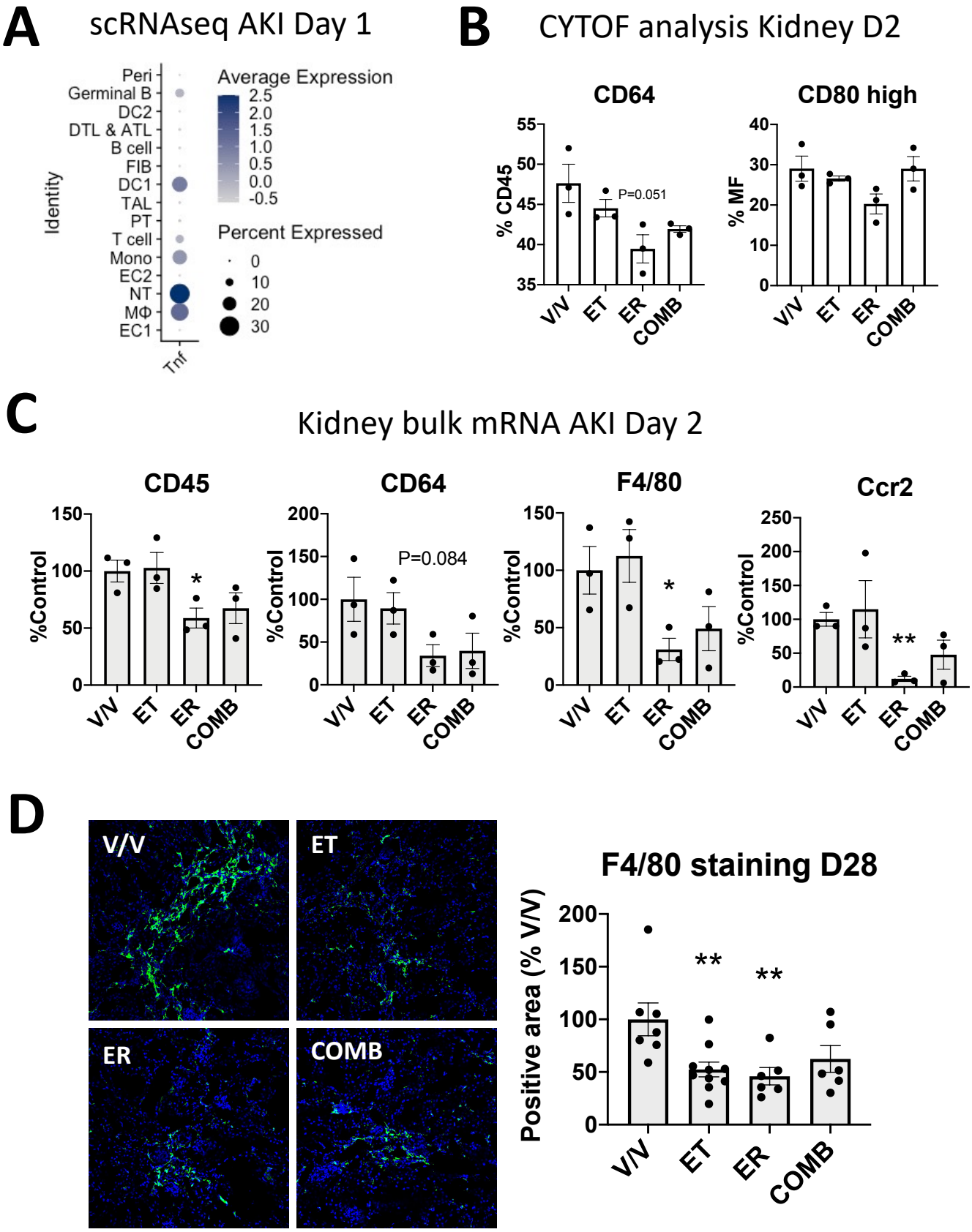



Figure 4: EGFR but not TNF inhibition reduces recruitment of immune cells into the injured kidney early after AKI.