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Foxp3$^+$ regulatory T cells participate in repair of ischemic acute kidney injury

Maria Teresa Gandolfo[1,2], Hye Ryoun Jang[2], Serena M. Bagnasco[1], Gang-Jee Ko[2], Patricia Agreda[2], Shailesh R. Satpute[2], Michael T. Crow[2], Landon S. King[2] and Hamid Rabb[2]

[1]Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD, USA and [2]Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD, USA

T lymphocytes modulate early ischemia-reperfusion injury in the kidney; however, their role during repair is unknown. We studied the role of TCRβ$^+$CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells (Tregs), known to blunt immune responses, in repair after ischemia-reperfusion injury to the kidney. Using a murine model of ischemic acute kidney injury we found that there was a significant trafficking of Tregs into the kidneys after 3 and 10 days. Post-ischemic kidneys had increased numbers of TCRβ$^+$CD4$^+$ and TCRβ$^+$CD8$^+$ T cells with enhanced pro-inflammatory cytokine production. Treg depletion starting 1 day after ischemic injury using anti-CD25 antibodies increased renal tubular damage, reduced tubular proliferation at both time points, enhanced infiltrating T lymphocyte cytokine production at 3 days and TNF-α generation by TCRβ$^+$CD4$^+$ T cells at 10 days. In separate mice, infusion of CD4$^+$CD25$^+$ Tregs 1 day after initial injury reduced INF-γ production by TCRβ$^+$CD4$^+$ T cells at 3 days, improved repair and reduced cytokine generation at 10 days. Treg manipulation had minimal effect on neutrophil and macrophage infiltration; Treg depletion worsened mortality and serum creatinine, while Treg infusion had a late beneficial effect on serum creatinine in bilateral ischemia. Our study demonstrates that Tregs infiltrate ischemic-reperfused kidneys during the healing process promoting repair, likely through modulation of pro-inflammatory cytokine production of other T cell subsets. Treg targeting could be a novel therapeutic approach to enhance recovery from ischemic acute kidney injury.

Correspondence: *Hamid Rabb, School of Medicine, Johns Hopkins University School of Medicine, Ross Research Building, Room 965, 720 Rutland Avenue, Baltimore, MD 21205, USA. E-mail: hrabb1@jhmi.edu*

Ischemia–reperfusion injury (IRI) is a major cause of acute kidney injury in native kidneys and the main cause of delayed graft function (DGF) in renal allograft recipients from deceased donors.[1–3] There is currently no specific therapy to either treat kidney IRI or accelerate healing. Mechanisms and mediators affecting the repair phase of renal IRI are largely unknown. Earlier studies have shown that T lymphocytes have a role in the early pathogenesis of renal IRI.[4–8] Recovery from IRI is a complex process, incompletely understood, and different T lymphocyte subpopulations could be involved in promoting damage or repair.[9,10] Isolation and transfer of kidney-infiltrating lymphocytes from ischemic-reperfused (IR) kidneys conferred partial protection from renal IRI, suggesting a beneficial role for these cells.[4] Alternatively, persistence of lymphocytes in the kidney after early injury might perpetuate damage in the extension phase of IRI and help explain the clinical association between DGF and increased occurrence of acute rejection.[1,2,11]

CD4$^+$CD25$^+$ T lymphocytes represent the most well-characterized subset of regulatory T cells (Tregs).[12] CD4$^+$CD25$^+$ Tregs have been implicated in preventing organ-specific autoimmunity and in modulating allogenic immune responses to induce graft tolerance in transplantation, both in experimental and in clinical settings.[13–15] Recently, nuclear transcription factor Foxp3 (forkhead box P3) has been shown not only to represent a specific marker for CD4$^+$CD25$^+$ Tregs, but also to regulate their development and function.[16,17] Tregs have shown to be protective in murine anti-glomerular basement membrane glomerulonephritis[18] and in adriamycin-induced nephropathy.[19] The presence and possible role of these cells in the repair phase of kidney IRI have not been explored yet.

The initial aim of this study was to characterize kidney-infiltrating T lymphocytes during healing from renal IRI in mice and to identify T subsets that could possibly mediate repair. We found that the healing phase was characterized by marked infiltration of TCRβ$^+$CD4$^+$CD25$^+$Foxp3$^+$ Tregs as well as increased trafficking of TCRβ$^+$CD4$^+$ and TCRβ$^+$CD8$^+$ T lymphocytes, characterized by enhanced pro-inflammatory cytokine production. We then tested the

hypothesis that $TCR\beta^+CD4^+CD25^+Foxp3^+$ Tregs could have a role in healing from kidney IRI. Treg depletion, starting 24 h after IRI, induced delayed histological evidence of repair, worsened mortality and serum creatinine (SCr). In complementary studies, transfer of $CD4^+CD25^+$ Tregs to mice 24 h after IRI promoted tissue healing and had a beneficial effect on SCr at late end points. The changes induced by Treg manipulation were associated with alterations in the production of pro-inflammatory cytokines from kidney-infiltrating T lymphocytes. Treg manipulation had minimal effects on kidney trafficking of other leukocyte populations.

RESULTS

Histological assessment of the healing phase of renal IRI

Histological damage and repair were assessed in IR kidneys compared with contralateral kidneys (CK), 3 and 10 days after 45 min unilateral ischemia. At each time point, CK demonstrated normal histology in cortex and outer medulla (Figure 1a and c) and low rate of tubular proliferation, less than 1%, by the percentage of Ki67-positive cells (Figure 1b and d). IR kidneys at 3 days showed severe structural damage, in cortex and outer medulla (Figure 1a). After 10 days, injury was milder in cortex, but still severe in outer medulla (Figure 1c). IR kidneys also showed increased tubular proliferative index with a peak at 3 days in both compartments, reaching values around 5–6% (Figure 1b). After 10 days, the proliferative index decreased to around 2%, but was still higher than CK (Figure 1d). Fibrosis was absent in CK at each end point and in IR kidneys at 3 days, and was minimal in IR kidneys at 10 days (data not shown).
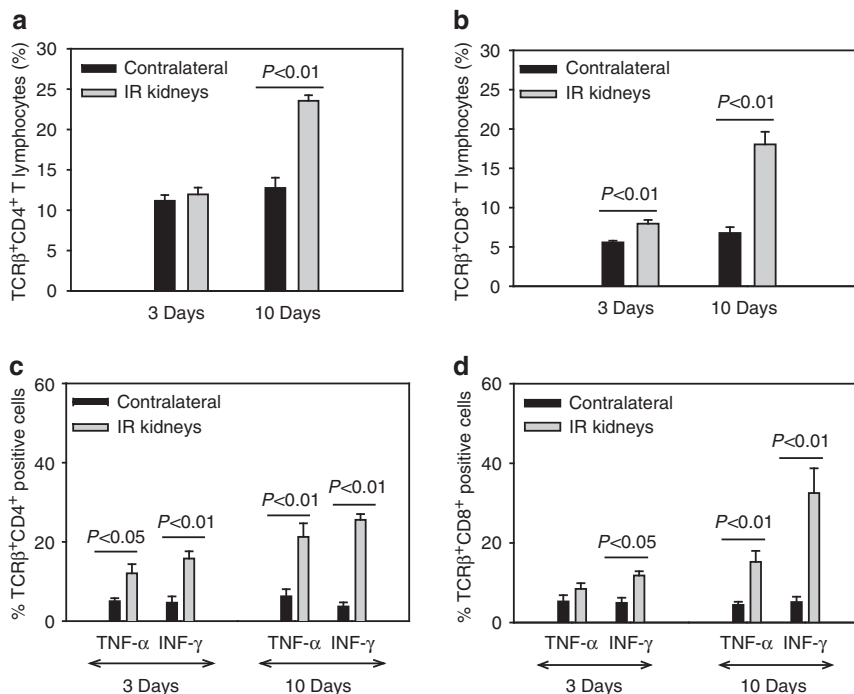
T lymphocytes infiltrated IR kidneys during the repair phase of renal IRI

During repair, increased number of kidney mononuclear cells (KMNCs) were isolated from IR kidneys compared with CK, both at 3 and 10 days after IRI (3 days: $7.37 \pm 0.63 \times 10^5$ versus $4.48 \pm 0.43 \times 10^5$, $P < 0.01$; 10 days: $4.23 \pm 0.18 \times 10^5$ versus $3.43 \pm 0.28 \times 10^5$, $P < 0.05$). Phenotyping KMNCs by flow cytometry analysis, we observed that T lymphocytes significantly infiltrated IR kidneys during the healing phase. Although increased presence of $TCR\beta^+CD4^+$ T lymphocytes was evident only at 10 days (Figure 2a), the trafficking of $TCR\beta^+CD8^+$ T cells was persistent during the healing phase (Figure 2b) (Total numbers of the T-cell subsets are shown in Supplementary Table S1a and b).

To elucidate the time-course of immune activation of renal T cells in the healing phase, cytokine production and CD69 expression were measured on $TCR\beta^+CD4^+$ and $TCR\beta^+CD8^+$ T lymphocytes. $TCR\beta^+CD4^+$ T cells infiltrating IR kidneys showed increased TNF-α and INF-γ production at 3 and 10 days after IRI (Figure 2c). Increased production of TNF-α at 10 days and of INF-γ at each end point was also observed for $TCR\beta^+CD8^+$ T lymphocytes infiltrating IR kidneys (Figure 2d). The percentage of $CD69^+$ among total $TCR\beta^+CD4^+$ and $TCR\beta^+CD8^+$ T cells was lower in IR kidneys compared with CK, after 3 days from IRI (% $TCR\beta^+CD4^+$ positive cells: 21.50 ± 1.55 versus $37.61 \pm 3.86\%$, $P = 0.01$; % $TCR\beta^+CD8^+$ positive cells: 25.68 ± 1.61 versus $32.99 \pm 2.50\%$, $P < 0.05$). In contrast, after 10 days, a pattern of novel cell activation was observed, with the percentage of $CD69^+$ among T lymphocytes being increased in IR kidneys (% $TCR\beta^+CD4^+$ positive cells:
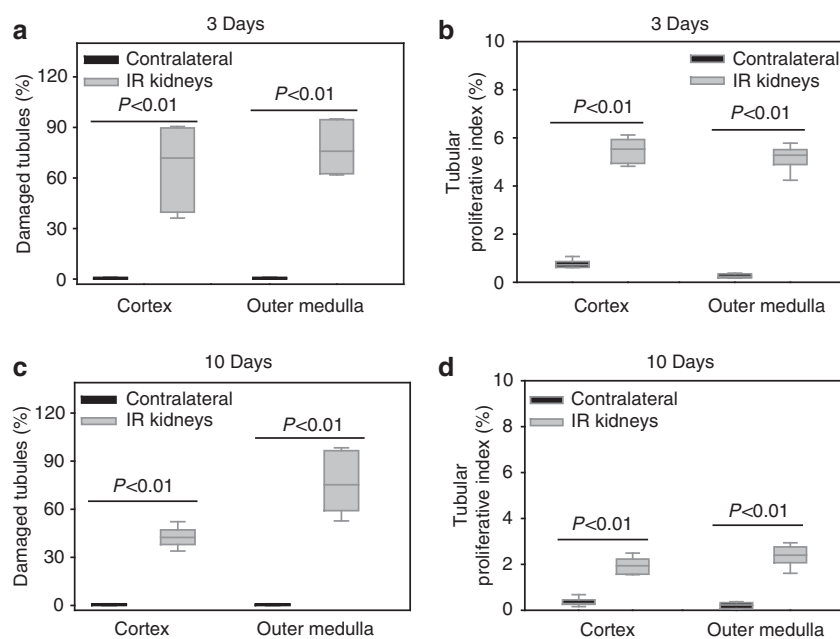
Figure 1 | Tubular damage and proliferation in the repair phase of renal IRI. Box plots show the 5th, 25th, 50th (median), 75th, and 95th percentile values for tubular damage score at 3 (**a**) and 10 days (**c**) after IRI in contralateral and IR kidneys and for tubular proliferative index at the same end points (**b**, **d**). $n = 6$/group.

Figure 2 | T lymphocyte infiltration in IR kidneys during repair. (**a**) The percentage of TCRβ$^+$CD4$^+$ T lymphocytes was increased in IR compared with contralateral kidneys, only at 10 days after IRI ($P<0.01$), as assessed by flow cytometry analysis. (**b**) The percentage of TCRβ$^+$CD8$^+$ T lymphocytes was increased in IR kidneys both at 3 and 10 days after IRI ($P<0.01$). (**c**) Production of TNF-α and INF-γ was increased during repair in TCRβ$^+$CD4$^+$ T lymphocytes infiltrating IR kidneys as compared with contralateral kidneys (TNF-α at 3 days: $P<0.05$; INF-γ at 3 days, TNF-α and INF-γ at 10 days: $P<0.01$), as assessed by intracellular staining and flow cytometry analysis. (**d**) At 3 days after IRI, production of INF-γ was increased in TCRβ$^+$CD8$^+$ T lymphocytes infiltrating IR kidneys as compared with contralateral kidneys ($P<0.05$); at 10 days, production of TNF-α and INF-γ was increased in TCRβ$^+$CD8$^+$ T lymphocytes infiltrating IR kidneys ($P<0.01$ for both cytokines). Means ± s.e. $n=6$/group.

60.43 ± 4.28 versus $43.96 \pm 5.33\%$, $P<0.05$; % TCRβ$^+$CD8$^+$ positive cells: 71.50 ± 2.30 versus $27.92 \pm 3.65\%$, $P=0.01$).

Kidney trafficking of Tregs was increased in the repair phase of renal IRI
We found that TCRβ$^+$CD4$^+$CD25$^+$Foxp3$^+$ Tregs were markedly infiltrating IR kidneys throughout the entire healing phase, compared with CK (Figure 3a) (Treg total numbers are shown in Supplementary Table S1c). Moreover, the percentage of the CD25$^+$Foxp3$^+$ Treg subset in the total kidney-infiltrating TCRβ$^+$CD4$^+$ T lymphocyte compartment was increased in IR kidneys at 3 and 10 days (Figure 3b). Foxp3$^+$ Treg staining by immunohistochemistry showed that more cells were present in IR kidneys, compared with CK, predominantly in the interstitium of cortex and outer medulla (3 days: 1.80 ± 0.37 versus 0.33 ± 0.21, $P<0.05$; 10 days: 2.60 ± 1.21 versus 0.17 ± 0.17 cells per 10 high power fields (HPF), $P<0.05$) (Figure 3c and d).

We also characterized other kidney-infiltrating leukocyte populations during repair after IRI. Neutrophils were increased in cortex and outer medulla at 3 and 10 days after IRI (Table 1.1a and 1b). CD11c$^+$F4/80$^-$ and CD11c$^+$F4/80$^+$ dendritic cells (DCs) and CD11c$^-$F4/80$^+$ macrophages were also augmented (Table 2.1a and 1b). TCRβ$^-$NK$^+$ natural killer (NK) cell infiltration into IR kidneys was

evident only at 3 days (Table 2.1a and 1b); however their activation status was increased at both end points (CD69 expression: 3 days 16.93 ± 1.23 versus $6.91 \pm 0.74\%$, $P<0.01$; 10 days: 15.25 ± 2.16 versus $5.64 \pm 0.61\%$, $P<0.01$).

***In vivo* Treg depletion delayed repair from renal IRI**
After identifying increased trafficking of Tregs into IR kidneys, we hypothesized a role for these cells during repair from kidney IRI. We initially depleted CD25$^+$ cells by PC61 monoclonal antibody (mAb) *in vivo*. The treatment was started 24 h after surgery to study repair more clearly without altering early injury. Mice were killed at 3 and 10 days. Efficacy of PC61 mAb treatment was checked in blood 24 h after the first injection (day 2) and in blood and spleen at death. PC61 mAb treatment led to a significant reduction of TCRβ$^+$CD4$^+$CD25$^+$Foxp3$^+$ Tregs (Table 3.1a and 2a). The percentage of TCRβ$^+$CD4$^+$Foxp3$^+$ Tregs, irrespective of CD25 surface expression, showed consistent, even though lower, reduction in depleted animals as well (Table 3.1b and 2b).

After 3 days, IR kidneys from PC61 mAb-treated mice showed increased tubular damage in outer medulla compared with controls (Figure 4a and e); these changes were associated with reduced tubular proliferation in cortex and outer medulla (Figure 4b and f). After 10 days, IR kidneys from
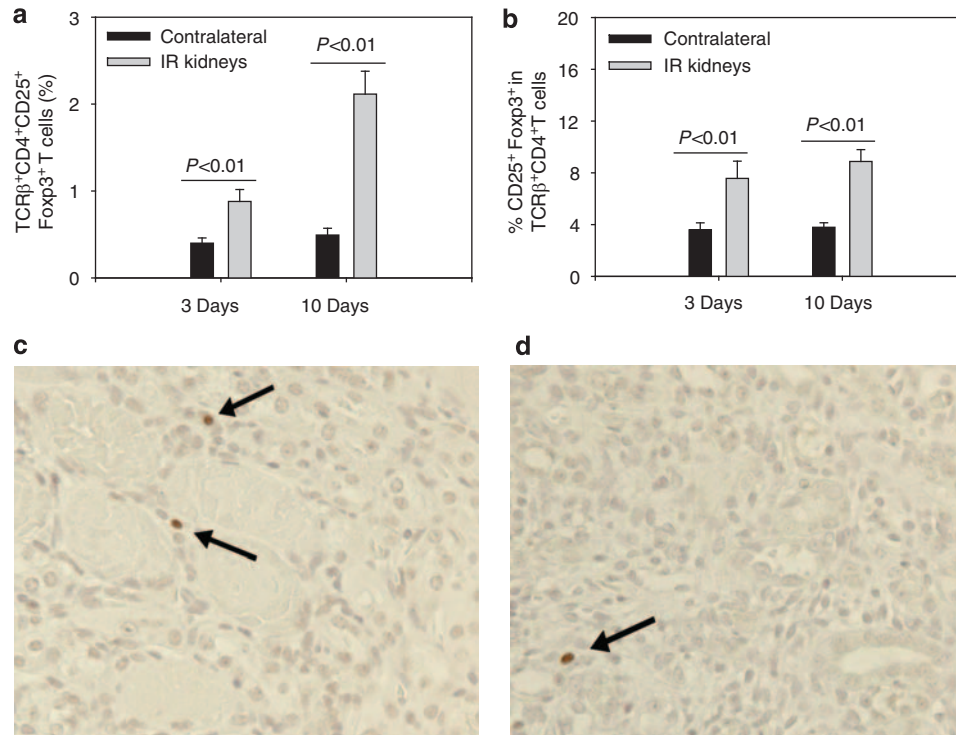
Figure 3 | Treg trafficking into IR kidneys during repair. (**a**) The percentage of TCRβ^+CD4$^+$CD25$^+$Foxp3$^+$ Tregs was increased in IR kidneys as compared with contralateral controls at 3 and 10 days after IRI, as assessed by flow cytometry analysis ($P < 0.01$). (**b**) The proportion of cells expressing both CD25 and Foxp3 among total TCRβ^+CD4$^+$ T lymphocytes was increased in IR kidneys, at each end point ($P < 0.01$). Means ± s.e. $n = 6$/group. (**c**, **d**) Representative images of Foxp3$^+$ cells infiltrating the interstitium of IR kidney outer medulla at 3 (**c**) and 10 days (**d**) after IRI (arrows). Sections were counterstained with hematoxylin (original magnification: ×200).

Table 1 | Neutrophil infiltration during repair from kidney IRI: effects of Treg manipulation

	Neutrophils (number/HPF)			
	a. 3 Days		b. 10 Days	
1.	**Contralateral**	**IR kidneys**	**Contralateral**	**IR kidneys**
Cortex	0.08 ± 0.08	5.75 ± 2.18*	0.08 ± 0.08	1.92 ± 0.63*
Outer medulla	0.17 ± 0.17	37.25 ± 2.99*	0.08 ± 0.08	13.08 ± 4.34*
2.	**Control IR kidneys**	**PC61 mAb-treated IR kidneys**	**Control IR kidneys**	**PC61 mAb-treated IR kidneys**
Cortex	1.73 ± 0.52	2.07 ± 0.53	7.25 ± 2.05	5.33 ± 1.38
Outer medulla	38.2 ± 3.95	35.13 ± 2.56	15.08 ± 3.11	11.25 ± 1.61
3.	**Control IR kidneys**	**CD4$^+$CD25$^+$ IR kidneys**	**Control IR kidneys**	**CD4$^+$CD25$^+$ IR kidneys**
Cortex	1.11 ± 0.37	1.22 ± 0.29	2.75 ± 0.77	3.83 ± 0.55
Outer medulla	28.56 ± 4.09	21.61 ± 3.49	9.92 ± 3.42	8.08 ± 1.04

Neutrophils were increased in cortex and outer medulla of IR kidneys compared with contralateral kidneys, both at 3 and 10 days after IRI (**1a** and **1b**). PC61 mAb treatment did not kidneys, induce changes in the total numbers of neutrophils infiltrating IR kidneys at each end point (**2a** and **2b**); in addition, Treg transfer did not affect neutrophil numbers at 3 and 10 days after IRI (**3a** and **3b**). Means ± s.e. n=4–6/group. *$P < 0.05$.

depleted mice showed persistent increased tubular damage, in cortex and outer medulla (Figure 4c and g), along with reduced cortical tubular proliferation (Figure 4d and h). Those data demonstrate a role for Tregs in promoting repair from kidney IRI. We also found that IR kidneys from PC61 mAb-treated animals had increased tissue levels of IL-2 and IL-10, but not IL-6, at 10 days after IRI, compared with controls (Supplementary Figure S1).

Treg depletion led to increased pro-inflammatory cytokine production from kidney T lymphocytes

We analyzed the effects of PC61 mAb treatment on kidney-infiltrating leukocytes. The percentage of IR kidney TCRβ^+CD4$^+$CD25$^+$Foxp3$^+$ Tregs was markedly reduced in depleted mice at both end points (Figure 5). The treatment did not affect the amount of KMNC infiltration or the percentage of TCRβ^+CD4$^+$ and TCRβ^+CD8$^+$ T cells

Table 2 | Infiltration of CD11c$^+$F4/80$^-$ and CD11c$^+$F4/80$^+$ dendritic cells (DCs), CD11c$^-$F4/80$^+$ macrophages, and TCRβ$^-$NK1.1$^+$ natural killer (NK) cells during repair from kidney IRI: effects of Treg manipulation

Cells (% gated)	a. 3 Days		b. 10 Days	
1.	Contralateral	IR kidneys	Contralateral	IR kidneys
CD11c$^+$F4/80$^-$	4.14 ± 0.19	17.28 ± 1.62**	6.53 ± 0.56	15.76 ± 1.74**
CD11c$^+$F4/80$^+$	5.39 ± 0.47	7.34 ± 0.62*	7.84 ± 0.94	11.33 ± 0.85*
CD11c$^-$F4/80$^+$	6.07 ± 0.65	9.04 ± 0.93*	6.48 ± 0.78	11.02 ± 1.28*
TCRβ$^-$NK1.1$^+$	3.89 ± 0.08	6.35 ± 0.46**	3.34 ± 0.38	5.14 ± 0.77
2.	Control IR kidneys	PC61 mAb-treated IR kidneys	Control IR kidneys	PC61 mAb-treated IR kidneys
CD11c$^+$F4/80$^-$	17.70 ± 1.25	18.15 ± 2.42	17.89 ± 0.32	19.21 ± 0.88
CD11c$^+$F4/80$^+$	5.47 ± 0.49	6.25 ± 0.89	11.92 ± 1.54	14.81 ± 0.47
CD11c$^-$F4/80$^+$	6.98 ± 0.57	8.08 ± 0.86	11.37 ± 0.90	13.38 ± 0.97
TCRβ$^-$NK1.1$^+$	7.43 ± 0.37	7.08 ± 0.80	6.38 ± 0.50	5.26 ± 0.30
3.	Control IR kidneys	CD4$^+$CD25$^+$ IR kidneys	Control IR kidneys	CD4$^+$CD25$^+$ IR kidneys
CD11c$^+$F4/80$^-$	13.68 ± 1.28	16.61 ± 1.46	17.76 ± 0.84	14.16 ± 0.46**
CD11c$^+$F4/80$^+$	5.15 ± 0.93	4.98 ± 0.38	9.59 ± 1.44	11.56 ± 1.33
CD11c$^-$F4/80$^+$	7.12 ± 1.15	5.93 ± 0.50	9.38 ± 1.14	10.86 ± 0.37
TCRβ$^-$NK1.1$^+$	7.69 ± 0.46	6.73 ± 0.45	5.90 ± 0.45	5.31 ± 0.21

Infiltration of CD11c$^+$F4/80$^-$ and CD11c$^+$F4/80$^+$ DCs and CD11c$^-$F4/80$^+$ macrophages was increased in IR kidneys both at 3 and 10 days after IRI compared with contralateral kidneys, as assessed by flow cytometry analysis; TCRβ$^-$NK1.1$^+$ NK cell infiltration was transiently augmented only at the 3-day end point (**1a** and **1b**). PC61 mAb treatment did not affect the percentages of cells infiltrating IR kidneys at each end point (**2a** and **2b**). Treg transfer was associated only with reduced infiltration of CD11c$^+$F4/80$^-$ DCs at 10 days after IRI (**3a** and **3b**). Means ± s.e. $n = 4$–6/group. *$P < 0.05$, **$P < 0.01$.

Table 3 | Efficacy of PC61 mAb treatment

|	a. TCRβ$^+$CD4$^+$CD25$^+$Foxp3$^+$ T cells (% gated)		b. TCRβ$^+$CD4$^+$Foxp3$^+$ T cells (% gated)	
	Control mice	PC61 mAb-treated mice	Control mice	PC61 mAb-treated mice
1. *3-day end point*				
Blood (day 2)	0.90 ± 0.07	0.14 ± 0.01***	1.22 ± 0.06	0.57 ± 0.04***
Blood (day 3)	0.69 ± 0.03	0.12 ± 0.01***	1.18 ± 0.06	0.65 ± 0.03***
Spleen (day 3)	0.86 ± 0.19	0.31 ± 0.04*	1.45 ± 0.20	0.92 ± 0.07**
2. *10-day end point*				
Blood (day 2)	0.78 ± 0.07	0.16 ± 0.02*	1.13 ± 0.03	0.61 ± 0.05*
Blood (day 10)	0.85 ± 0.18	0.07 ± 0.01***	1.40 ± 0.16	0.42 ± 0.03***
Spleen (day 10)	1.67 ± 0.11	0.46 ± 0.05***	2.13 ± 0.14	1.19 ± 0.09***

Treg depletion was evaluated in blood at day 2 after IRI, in blood and spleen at killing, both for the 3- and 10-day end point. The percentage of TCRβ$^+$CD4$^+$CD25$^+$Foxp3$^+$ Tregs was markedly reduced in mice treated with PC61 mAb (**1a** and **2a**). In addition, the percentage of TCRβ$^+$CD4$^+$Foxp3$^+$ Tregs, irrespective of CD25 expression, was reduced after PC61 mAb treatment, although to a smaller extent (**1b** and **2b**). Means ± s.e. *$P < 0.05$, **$P = 0.05$, ***$P < 0.01$.

(3 days: $P = 1.00$, $P = 0.14$ and $P = 0.58$; 10 days: $P = 0.88$, $P = 0.25$ and $P = 0.89$). There was no change in the trafficking of neutrophils, DCs, macrophages and NK cells at each time point (Table 1.2a and 2b, 2.2a and 2b). PC61 mAb treatment was associated with increased TCRβ$^+$CD8$^+$ T and NK cell CD69 expression at 3 days (% TCRβ$^+$CD8$^+$ positive cells: 23.22 ± 0.91 versus 19.21 ± 1.23, $P < 0.05$; % TCRβ$^-$NK1.1$^+$ NK positive cells: 19.01 ± 0.36 versus 13.67 ± 1.28%, $P = 0.01$), but not at 10 days ($P = 0.12$, $P = 0.63$). TCRβ$^+$CD4$^+$ T cell CD69 expression was not affected by CD25 depletion at 3 and 10 days ($P = 0.46$ and $P = 0.78$). TNF-α and INF-γ production from renal T lymphocytes was markedly increased in IR kidneys from depleted mice at 3 days (Figure 6a–d). TNF-α, but not INF-γ, production from TCRβ$^+$CD4$^+$ T lymphocytes was also markedly augmented in PC61 mAb-treated IR kidneys at

10 days (Figure 6e–f). Cytokine generation from infiltrating TCRβ$^+$CD8$^+$ T cells was similar between the groups at 10 days (TNF-α: $P = 0.47$, INF-γ: $P = 0.49$).

Transfer of CD4$^+$CD25$^+$ Tregs promoted kidney repair after renal IRI

Subsequently, 1.2×10^6 CD4$^+$CD25$^+$ Tregs were isolated and purified from spleens of C57BL/6J mice and then injected i.v. into ischemic mice, 24 h after IRI. At day 3 after IRI, no changes in histological damage and tubular regeneration were observed in IR kidneys from transferred mice (percentage of damaged tubules: cortex $P = 0.17$, outer medulla $P = 0.35$; tubular proliferative index: cortex $P = 0.12$, outer medulla $P = 0.46$). However, after 10 days, mice that had received CD4$^+$CD25$^+$ Treg transfer showed markedly reduced tubular damage in the cortex and outer medulla
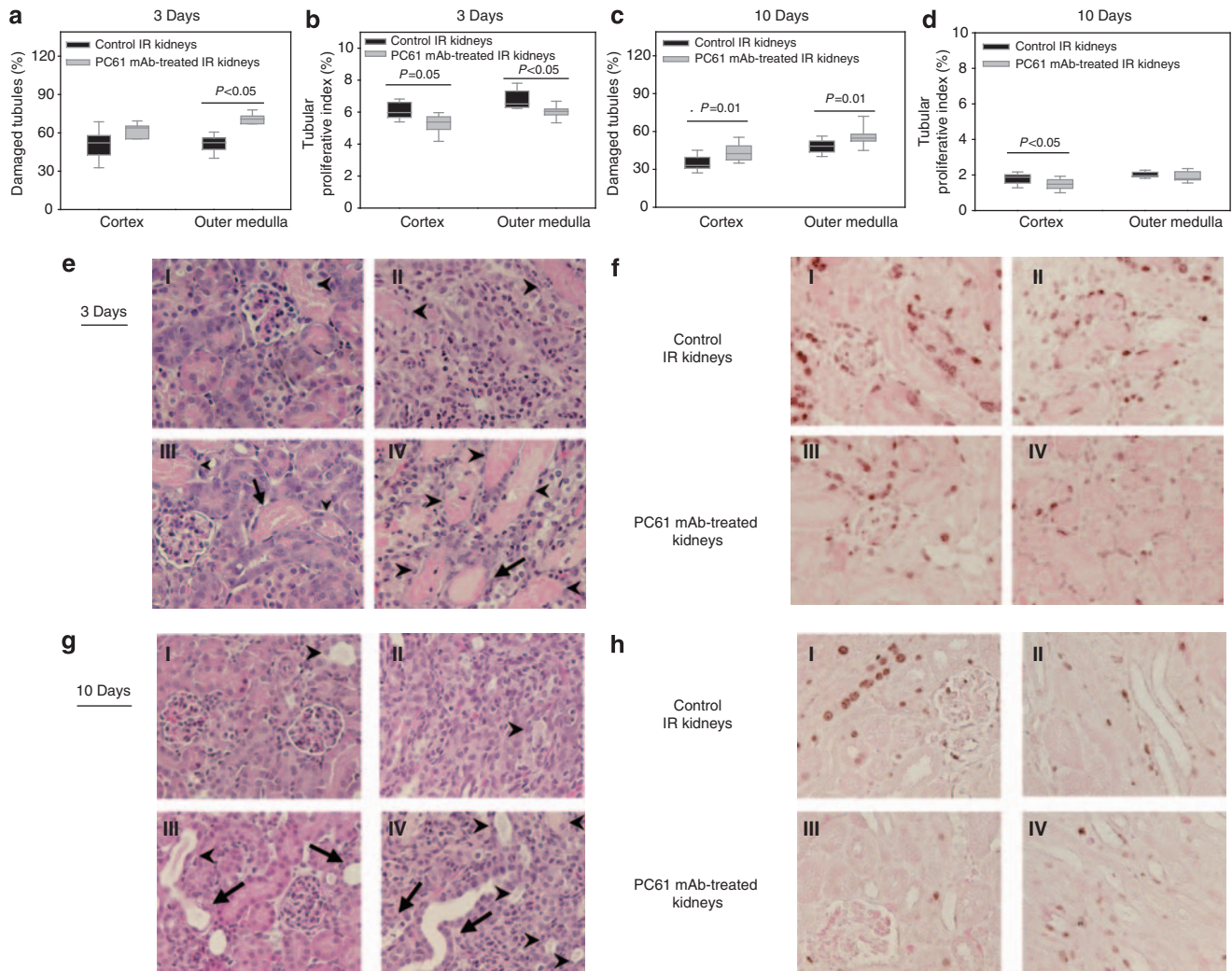
Figure 4 | Effects of Treg depletion on tubular damage and proliferation during repair from kidney IRI. (a–d) Box plots show the 5th, 25th, 50th (median), 75th, and 95th percentile values for tubular damage score and tubular proliferative index at 3 (**a** and **b**) and 10 (**c** and **d**) days in IR kidneys obtained from mice treated with isotype control (Control IR kidneys) or with PC61 mAb (PC61 mAb-treated IR kidneys), starting 24 h after IRI. PC61 mAb-treated IR kidneys had increased tubular damage in outer medulla ($P < 0.05$) and reduced tubular regeneration in cortex and outer medulla ($P \leqslant 0.05$) compared with controls, at 3 days ($n = 5$–6/group). IR kidneys from PC61 mAb-treated mice showed increased tubular damage in cortex and outer medulla ($P = 0.01$) ($n = 12$/group), along with reduced cortical tubular regeneration at 10 days ($P < 0.05$) ($n = 9$/group). (**e**, **g**) Representative histological images of hematoxylin and eosin–stained renal sections at 3 days (**e**) and 10 days (**g**) for control IR kidneys (cortex (**I**), outer medulla (**II**)) and PC61 mAb-treated IR kidneys (cortex (**III**), outer medulla (**IV**)). Tubular injury is identified by diffuse tubular dilatation (arrows), intraluminal casts (arrowheads), and tubular cell detachment. (**f**, **h**) Representative images of Ki67 staining on kidney tissues at 3 days (**f**) and 10 days (**h**) for control IR kidneys (cortex (**I**), outer medulla (**II**)) and PC61 mAb-treated IR kidneys (cortex (**III**), outer medulla (**IV**)). Here, sections were counterstained with Eosin (original magnification: ×200).
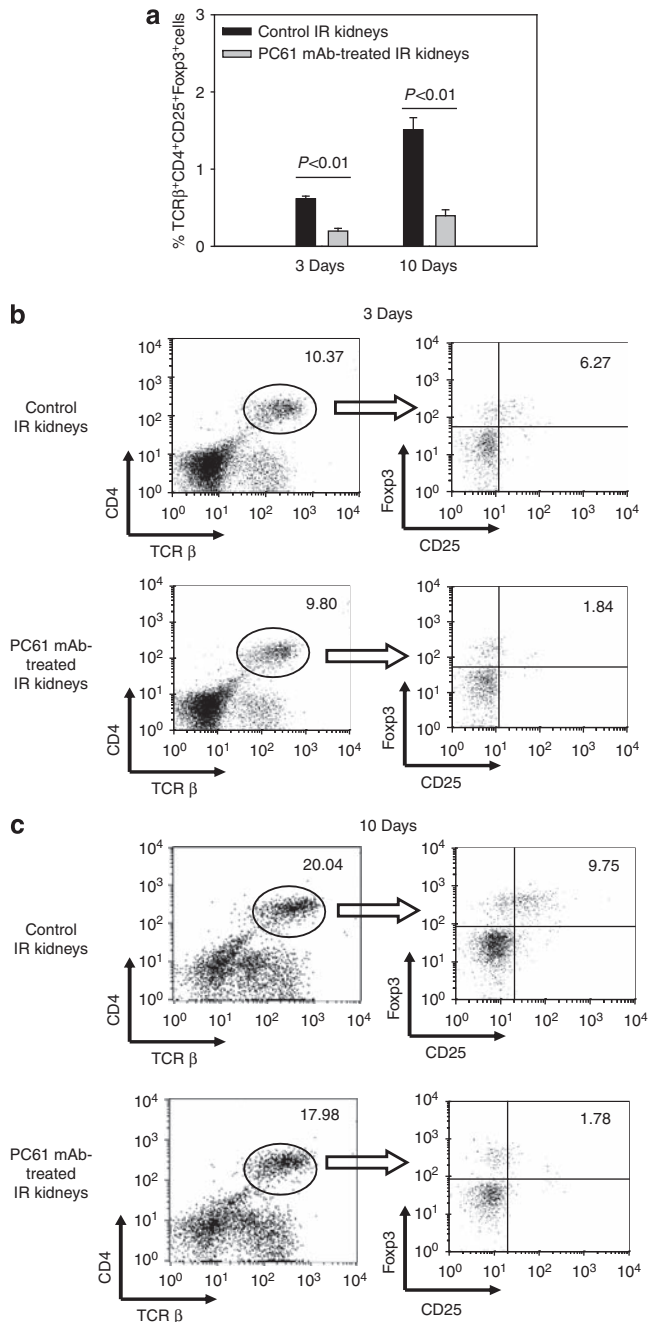
of IR kidneys, compared with control ischemic mice (Figure 7a). Tubular regeneration was increased in Treg transferred mice in both compartments (Figure 7b).

CD4+CD25+ Treg transfer was associated with reduced production of pro-inflammatory cytokines by kidney-infiltrating T lymphocytes

Mice receiving $CD4^+CD25^+$ Treg transfer had increased KMNC infiltration in IR kidneys at 3 days ($8.46 \pm 0.62 \times 10^5$ versus $6.60 \pm 0.48 \times 10^5$, $P < 0.05$) with no differences in the percentage of T-cell subsets (data not shown), other leukocyte populations (Table 1.3a and 2.3a) or activation status of

T and NK cells (data not shown). However, $TCR\beta^+CD4^+$ T cells infiltrating IR kidneys from transferred mice showed reduced INF-γ, but not TNF-α, generation at 3 days (percentage of positive cells: 8.57 ± 1.98 versus 15.67 ± 2.09, $P < 0.05$). Cytokine generation from $TCR\beta^+$ $CD8^+$ T lymphocytes was unchanged (TNF-α: $P = 0.85$, INF-γ: $P = 0.20$).

At 10 days, persistent increase of KMNCs was observed (Figure 8a), without changes in T-cell populations ($TCR\beta^+CD4^+$ $P = 0.72$, $TCR\beta^+CD8^+$ $P = 0.47$, $TCR\beta^+$ $CD4^+CD25^+Foxp3^+$ $P = 0.13$). A significant increase in the proportion of the Treg subset in the $TCR\beta^+CD4^+$

Figure 5 | Percentage of kidney-infiltrating TCRβ+CD4+CD25+Foxp3+ Tregs after PC61 mAb treatment. (a) The percentage of TCRβ+CD4+CD25+Foxp3+ Tregs was decreased in IR kidneys from PC61 mAb-treated mice, compared with control IR kidneys after 3 and 10 days, as assessed by flow cytometry analysis ($P < 0.01$). Means ± s.e. $n = 5$–6/group. **(b, c)** Representative dot plots from control and PC61 mAb-treated IR kidneys at the 3 **(b)** and 10 day **(c)** end points. TCRβ+CD4+ T lymphocytes were initially gated from the lymphocyte area (and are identified by the circle in the dot plots on the left) and subsequently the expression of CD25 and Foxp3 was examined in this population. The percentage of TCRβ+CD4+CD25+Foxp3+ Tregs was obtained by multiplying the percentage of TCRβ+CD4+ T lymphocytes by the percentage of CD25+Foxp3+ cells (upper right quadrant in the dot plots on the right).

T compartment was present in the transferred mice at day 10 (Figure 8b). A reduced percentage of CD11c+F4/80− DCs infiltrated IR kidneys at 10 days in Treg-transferred mice, whereas the other leukocyte populations examined did not change (Table 1.3b and 2.3b). No difference was observed in activated TCRβ+CD4+ and TCRβ+CD8+ T lymphocytes and NK cells ($P = 0.28$, $P = 0.83$ and $P = 0.92$). TCRβ+CD4+ T lymphocytes infiltrating IR kidneys of transferred mice showed reduced TNF-α and INF-γ generation and TCRβ+CD8+ T cells diminished INF-γ production compared with control mice, at 10 days (Figure 8c–f).

Treg manipulation was associated with changes in TNF-α kidney tissue levels

Increased TNF-α tissue levels characterized IR kidneys throughout the repair phase (Table 4.1a and 1b). PC61 mAb-treated IR kidneys showed increased TNF-α levels both at 3 and 10 days after IRI, compared with controls (Table 4.2a and 2b). In contrast, IR kidneys from transferred mice had reduced TNF-α tissue levels at day 10 (Table 4.3a and 3b).

Treg manipulation affected kidney function during repair of kidney IRI

To study the effects of Treg manipulation on kidney function during healing after IRI, we decreased ischemia times to 30 min and performed bilateral pedicle clamping. CD25 depletion, 24 h after IRI, increased SCr at day 2 and reduced survival rates compared with isotype control administration (Figure 9a and b). Treg transfer reduced SCr at day 7 without effects on mortality (Figure 9c).

DISCUSSION

These data demonstrate that increased trafficking of T lymphocytes, particularly Tregs, occurs during repair from kidney IRI. More importantly, using complementary depletion and transfer techniques, we found that kidney-infiltrating TCRβ+CD4+CD25+Foxp3+ Tregs have a direct role in promoting healing. A likely mechanism of the beneficial effect is by negative modulation of the pro-inflammatory cytokines produced by other T lymphocyte subsets.

T lymphocyte infiltration has been described as an early and transient (1–3 h) event after renal IRI (hit and run hypothesis), with cells disappearing by 24 h.[4,5] There have been limited publications demonstrating the presence of T lymphocytes during healing and only immunohistochemistry was used.[7,9,10,20] Our current study provides new information on T lymphocyte trafficking in the repair phase of IRI, using cell isolation from the kidney and cell subset phenotyping by flow cytometry analysis. Our data demonstrated that renal infiltration of TCRβ+CD8+ and TCRβ+CD4+ T lymphocytes was increased during repair. Previous reports have linked the upregulated production of Th1 pro-inflammatory cytokines by T lymphocytes to the early pathogenesis of kidney IRI.[4,21–23] We demonstrated that, in the repair phase of IRI, T lymphocytes showed persistent enhanced ability to produce the pro-inflammatory Th1
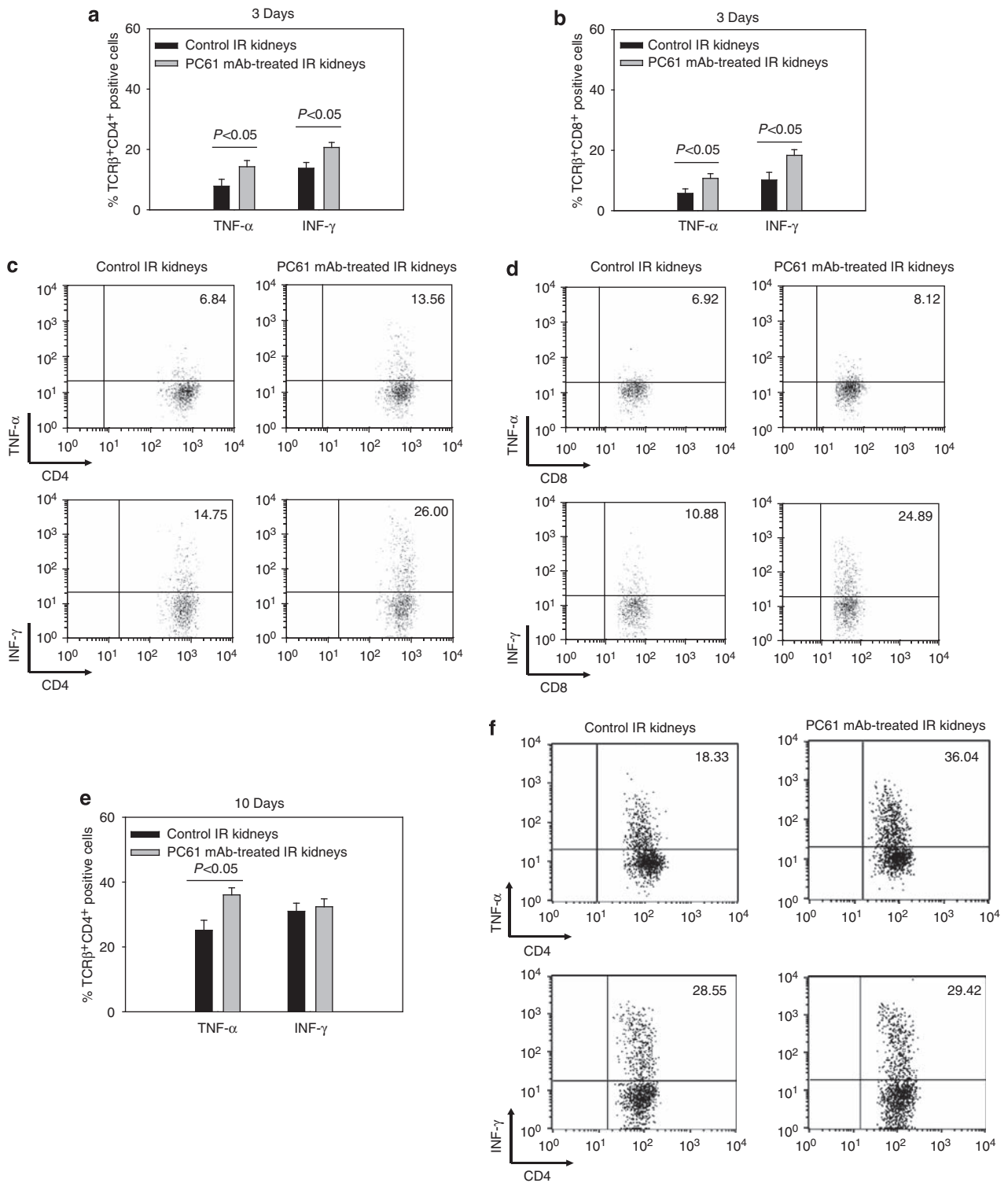
Figure 6│Effects of Treg depletion on the pro-inflammatory cytokine production from kidney-infiltrating T lymphocytes.
(**a**, **b**) TNF-α and INF-γ production from TCRβ$^+$CD4$^+$ (**a**) and TCRβ$^+$CD8$^+$ (**b**) T lymphocyte count was markedly increased in IR kidneys from PC61 mAb-treated mice as compared with controls at day 3 ($P < 0.05$), as assessed by intracellular staining and flow cytometry analysis. Means ± s.e. $n = 6$/group. (**c**, **d**) Representative dot plots from control and PC61 mAb-treated IR kidneys. TCRβ$^+$CD4$^+$ (**c**) and TCRβ$^+$CD8$^+$ (**d**) T lymphocytes were initially gated as described in Figure 5 (dot plot not presented), then the percentage of T lymphocyte subsets that were positive for TNF-α and INF-γ was determined as shown (upper right quadrant) for each group. (**e**) TNF-α production from TCRβ$^+$CD4$^+$ T lymphocytes was markedly increased in IR kidneys from PC61 mAb-treated mice as compared with controls at 10 days ($P < 0.05$), as assessed by intracellular staining and flow cytometry analysis; INF-γ production was not affected by PC61 mAb treatment. Means ± s.e. $n = 6$/group. (**f**) Representative dot plots from control and PC61 mAb-treated IR kidneys.

a

b
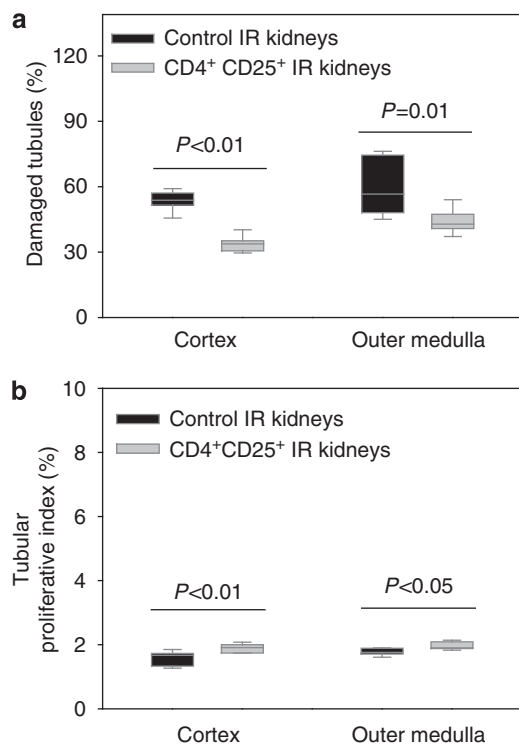
Figure 7 | **Promotion of kidney repair by CD4⁺CD25⁺ Treg transfer, 10 days after IRI.** (**a**, **b**) Box plots show the 5th, 25th, 50th (median), 75th, and 95th percentile values for tubular damage score (**a**) and tubular proliferative index (**b**) at 10 days in IR kidneys obtained from mice treated with saline (control IR kidneys) or subjected to Treg transfer 24 h after IRI (CD4⁺CD25⁺ IR kidneys). CD4⁺CD25⁺ IR kidneys presented reduced tubular damage (cortex: $P<0.01$; outer medulla: $P=0.01$) and increased tubular proliferation (cortex: $P<0.01$; outer medulla: $P<0.05$), 10 days after the surgery. $n=7$/group.

cytokines TNF-α and INF-γ. CD69 is one of the earliest molecules to appear on activated T cells with maximum levels of surface expression at 16–24 h after antigen stimulation.[24] We found that the state of recent activation of T lymphocytes changed with healing, being lower at 3 days and increasing later on. These data suggest a biphasic response, with a delayed wave of T-cell activation, possibly induced by release of auto-antigens not previously exposed. Ischemia *per se* can induce increased kidney immunogenicity,[10,25] and later onset of novel T-cell activation during recovery from IRI could contribute to the higher risk of rejection observed in patients who experience DGF.[2]

CD4⁺CD25⁺Foxp3⁺ Tregs are key regulators in auto-immunity and transplant tolerance;[12] these cells can suppress innate and cognate immune responses, using multiple mechanisms.[26–28] Expression of pro-inflammatory cytokines is potently suppressed by Tregs.[29,30] The repair phase of kidney IRI, in this study, was characterized by increased infiltration of TCRβ⁺CD4⁺CD25⁺Foxp3⁺ Tregs; moreover, a preferential recruitment of the regulatory subset was suggested by the increased proportion of cells expressing both

CD25 and Foxp3 in the total TCRβ⁺CD4⁺ T lymphocyte compartment in IR kidneys. In patients with renal transplant rejection, Foxp3⁺ Tregs markedly infiltrate the graft, probably limiting anti-allograft immunity and protecting the graft from the activity of effector T cells.[31] In SCID mice, transfer of CD4⁺CD25⁺ Tregs significantly reduced chronic damage from adriamycin-induced nephropathy, through inhibition of macrophage activity.[32] In our study, we hypothesized that Tregs could have a role in repair from kidney IRI by counterbalancing pathogenic lymphocyte effector responses.

We first depleted Tregs by administration of anti-CD25 mAbs, using an established approach,[19,33] because Foxp3 is an intracellular transcription factor and depleting Foxp3⁺ cells is not directly achievable. When we checked the depletion rate of TCRβ⁺CD4⁺Foxp3⁺ Tregs in blood and spleen, irrespective of CD25 expression, we observed that it was lower compared with the depletion rate of TCRβ⁺CD4⁺CD25⁺Foxp3⁺ Tregs. These data were in accordance with previous reports showing that the principal mechanism of action of PC61 mAb is by inducing Treg functional inactivation, rather than complete depletion, and that only part of the Foxp3-expressing cells is eliminated.[34] In kidney, Treg depletion led to worse histological damage and delayed tubular regeneration both at 3 and 10 days after IRI.

Marked increase in pro-inflammatory cytokine production characterized T cells infiltrating IR kidneys of depleted mice at 3 days; moreover, at the later end point, TNF-α production, but not INF-γ, was markedly increased in TCRβ⁺CD4⁺ T cells. We also evaluated the effects of Treg depletion on trafficking of other leukocyte populations and did not observe changes in number of neutrophils, DCs, macrophages or NK cells at each time point. Nevertheless, NK cell activation was increased at 3 days, suggesting a potential involvement of these cells in the delayed repair associated with Treg depletion.

Using a complementary approach to elucidate if Tregs had a beneficial role in repair after IRI, we transferred CD4⁺CD25⁺ Tregs in mice 24 h after IRI. Wild-type mice, with a normal immune system, were chosen as recipients, because our hypothesis implied that Tregs could positively affect repair by inhibiting cytokine production from other T lymphocytes. Treg transfer did not affect histology at 3 days, but it was associated with reduced tubular damage and increased proliferation at 10 days. Increased amount of KMNCs infiltrating the kidneys was observed at both end points, but higher percentage of CD25⁺Foxp3⁺ cells in the renal TCRβ⁺CD4⁺ T subset was evident only at 10 days, suggesting a slower and time-dependent change in the proportion of infiltrating subsets.

In accordance with our hypothesis, at 3 days, even in the absence of histological changes, INF-γ production was reduced in renal TCRβ⁺CD4⁺ T cells from the transferred mice; at 10 days, production of pro-inflammatory cytokines by T lymphocytes was markedly diminished. Reduced infiltration of CD11c⁺F4/80⁻ DCs also characterized the
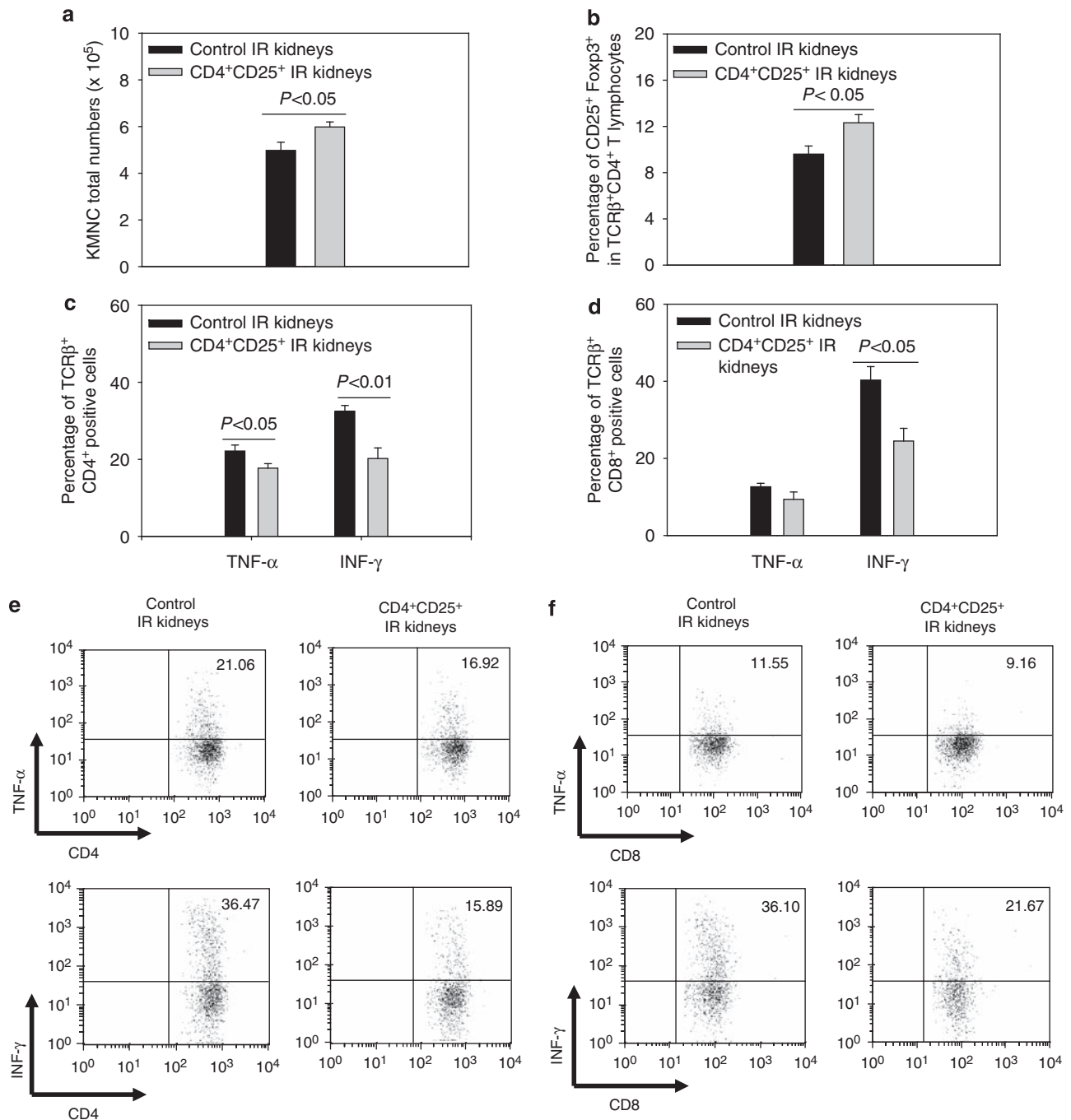
Figure 8 | Effects of Treg transfer on kidney-infiltrating lymphocytes, 10 days after IRI. (**a**) Increased numbers of mononuclear cells infiltrating the kidney (KMNCs) were observed in IR kidneys from mice subjected to Treg transfer 24 h after IRI (CD4$^+$CD25$^+$ IR kidneys), as compared to mice treated with saline (control IR kidneys) ($P < 0.05$). (**b**) The relative abundance of CD25$^+$Foxp3$^+$ cells in the TCRβ$^+$CD4$^+$ T subpopulation was increased in CD4$^+$CD25$^+$ IR kidneys ($P < 0.05$). (**c**) The production of TNF-α and INF-γ from TCRβ$^+$CD4$^+$ T lymphocytes was reduced in CD4$^+$CD25$^+$ IR kidneys as compared with controls ($P < 0.05$ and $P < 0.01$, respectively), whereas (**d**) only INF-γ production from TCRβ$^+$CD8$^+$ T lymphocytes was reduced ($P < 0.05$). Means ± s.e. $n = 6$/group. (**e, f**). Representative dot plots from control and CD4$^+$CD25$^+$ IR kidneys, showing the percentage of TCRβ$^+$CD4$^+$ T lymphocytes (**e**) and TCRβ$^+$CD8$^+$ T lymphocytes (**f**) that were positive for TNF-α and for INF-γ (upper right quadrants) in each group.

later end point suggesting that these cells are a potential target of Treg modulation in our model. CD69 expression on TCRβ$^+$CD4$^+$ T lymphocytes was unaffected by Treg manipulation. Previous *in vitro* data[35] suggested that Tregs

are unable to inhibit the initial events (upregulation of CD25 and CD69) of target CD4$^+$ T-cell activation.

Kidney TNF-α levels changed in a fashion similar to the degree of tissue injury, suggesting that a continuum could

Table 4 | TNF-α kidney tissue levels during repair of renal IRI: effects of Treg manipulation

	TNF-α kidney levels (pg/mg of whole kidney protein extract)			
	a. 3 Days		**b. 10 Days**	
1.	Contralateral	IR kidneys	Contralateral	IR kidneys
	4.68 ± 0.69	6.71 ± 0.60*	3.44 ± 0.42	13.44 ± 1.70**
2.	Control IR kidneys	PC61 mAb-treated IR kidneys	Control IR kidneys	PC61 mAb-treated IR kidneys
	5.26 ± 0.69	9.62 ± 1.65*	8.33 ± 0.86	13.38 ± 2.11*
3.	Control IR kidneys	CD4+CD25+ IR kidneys	Control IR kidneys	CD4+CD25+ IR kidneys
	9.03 ± 1.27	9.35 ± 1.34	16.49 ± 1.63	10.37 ± 1.17*

TNF-α kidney tissue levels were increased in IR kidneys as compared with contralateral kidneys at both 3 and 10 days after IRI (**1a and 1b**). PC61 mAb treatment increased TNF-α tissue levels in IR kidneys at each end point (**2a and 2b**). Treg transfer induced reduction in TNF-α tissue levels in IR kidneys only at 10 days (**3a and 3b**). Means ± s.e. $n = 5$–10/group. *$P < 0.05$, **$P < 0.01$.
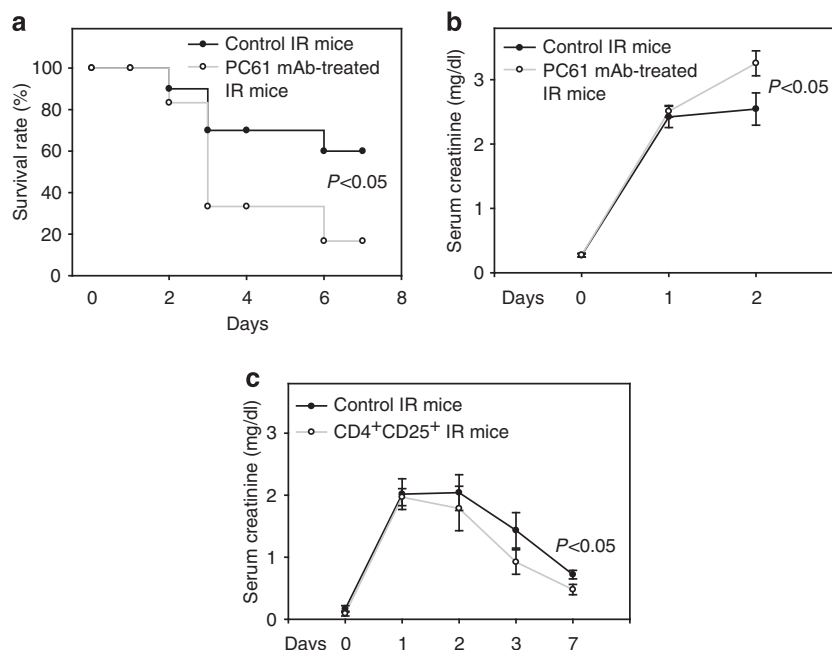
Figure 9 | Treg manipulation starting 24 h after injury affected kidney function during repair of IRI. (a, b) Using 30 min bilateral ischemia, Treg depletion, started 24 h after IRI, worsened survival rate (**a**) and increased serum creatinine levels (**b**) ($P < 0.05$), as compared with isotype control administration ($n = 18$–20/group at day 0). A higher mortality rate in the Treg-depleted mice limited proper comparison of serum creatinine levels with the control group after day 2. (**c**) Mice receiving Treg transfer, 24 h after IRI, had reduced serum creatinine levels at day 7 as compared with saline controls ($P < 0.05$) (CD4+CD25+ IR mice: $n = 8$ at day 0, 1 and 2; $n = 7$ from day 3; control IR mice: $n = 10$ at day 0, 1 and 2; $n = 8$ from day 3; survival rate $P = 0.68$).

exist among T lymphocyte TNF-α production, tissue levels, and histological damage.

It is important to point out that the effects of Treg depletion and transfer on histology and T lymphocyte cytokine production were different according to the time point considered. Although Treg depletion was effective at 3 days after IRI, Treg transfer was associated with histological changes only at 10 days, even if reduced cytokine production was present earlier. These observations suggest that Treg transfer requires more time to become effective. We also conducted preliminary studies to test if Treg manipulation could affect kidney function, switching to a well-validated bilateral 30 min IRI model. CD25 depletion worsened SCr within 1 day of mAb administration and increased mortality,

whereas Treg transfer induced improvement of kidney function at a later end point.

In summary, our findings identify a novel role for Tregs as mediators of kidney repair after IRI. As there is no specific therapy available to accelerate healing during acute kidney injury and DGF, these data point to the exciting potential of harnessing Tregs for therapeutics in kidney IRI.

MATERIALS AND METHODS
Mice
Male C57BL/6J mice, 6–12 weeks old (The Jackson Laboratory, Bar Harbor, ME), were housed under specific pathogen-free conditions. All experiments were performed in accordance with the institutional Animal Care and Use Committee guidelines.

Renal IRI model

An established model[36] of renal IRI was used; during the surgical procedure, the left renal pedicle was clamped for 45 min, followed by reperfusion. The animals were killed at 3 or 10 days after IRI. The unilateral model was chosen to prevent mortality of mice. In a different set of experiments, mice underwent 30 min bilateral ischemia, followed by reperfusion, and were killed after 7 days.

Histology

Histological damage at 3 and 10 days was evaluated on hematoxylin and eosin and Masson's Trichrome-stained kidney sections (4 μm). Damaged tubules were identified by the presence of diffuse tubular dilatation, intraluminal casts and/or tubular cell blebbing, vacuolization and detachment, in cortex and outer medulla in 6–10 HPF ($\times$ 400 magnification) per hematoxylin and eosin section, in a blind fashion. The number of damaged tubules was divided by the number of the total tubules in the same field to obtain the '% damaged tubules'. No histological damage was observed in the papilla. The presence of fibrosis was evaluated on Masson's Trichrome-stained slides.

Neutrophil count

Neutrophils were counted in cortex and outer medulla in at least six HPF for each hematoxylin and eosin section[37] and expressed as numbers/HPF.

Immunohistochemistry

Staining of Ki67[38] (clone TEC-3, DAKO Carpinteria, CA, USA) and Foxp3[39] (Clone FJK-16s, eBioscience, San Diego, CA, USA) proteins was performed on formalin-fixed kidney sections (details in Supplementary Information).

Lymphocyte isolation from mouse kidneys

At 3 and 10 days, mice were exsanguinated and kidneys collected and decapsulated. Experiments were performed by pooling two kidneys together, for each analysis. Viable kidney-infiltrating KMNCs were isolated as previously described[4] and counted on a hemocytometer by trypan blue exclusion. The percentage of infiltrating leukocyte subpopulations into IR and contralateral kidneys was determined by flow cytometry analysis.

Antibodies

Reagents and mAbs for flow cytometry analysis were obtained from BD Biosciences (San José, CA, USA), Invitrogen (Carlsbad, CA, USA) and eBioscience (details in Supplementary Information).

Flow cytometry analysis

Surface and intracellular staining of KMNCs was performed as previously described.[4,23] Multiple-color immunofluorescence staining was analyzed using FACSCalibur and LSR II instruments and CellQuest (BD Biosciences) and FSC Express softwares (DeNovo Software, Los Angeles, CA, USA) (details in Supplementary Material).

In vivo Treg depletion

Treg depletion was achieved by i.p. injection of 500 μg of anti-CD25 mAb (PC61, BioXCell, West Lebanon, NH, USA) every 72 h, starting 24 h after surgery; in control mice, appropriate isotype control (BioXCell) was administered. Animals were killed at 3, 7 and 10 days after IRI. CD25 depletion was confirmed by flow cytometry analysis, using anti-CD25 mAb (7D4), in blood samples at day 2 and in blood and spleen samples at the time of killing.

Isolation and purification of CD4⁺CD25⁺ Tregs

$CD4^+CD25^+$ Tregs were isolated from spleens of C57BL/6J mice using magnetic beads separation ($CD4^+CD25^+$ regulatory T cell kit; Miltenyi Biotec Inc., Auburn, CA, USA). The purity of the population was confirmed by flow cytometry analysis and routinely reached $>90\%$.

Adoptive transfer of CD4⁺CD25⁺ Tregs in IR mice

After 24 h from renal IRI, mice were injected with 1.2×10^6 $CD4^+CD25^+$ Tregs (or equal volumes of 0.9% saline solution as control) via the tail vein. Mice were killed at 3, 7 and 10 days.

TNF-α renal tissue levels

TNF-α kidney tissue levels were measured in each experimental group at 3 and 10 days, by ELISA (Biolegend, San Diego, CA, USA).

Assessment of renal function

SCr levels were measured from blood samples collected at baseline and at day 1, 2, 3 and 7 after renal bilateral IRI, using a commercial creatinine kit (Pointe Scientific, Canton, MI, USA) and an autoanalyzer (Roche, Indianapolis, IN, USA).

Statistical analysis

Statistical comparisons between groups were performed by Student's *t*-test and, for non-parametric data sets, by Mann–Whitney *U*-test. A *P*-value $\leqslant 0.05$ was considered statistically significant.

SUPPLEMENTARY MATERIAL

Table S1. Total number of T cell subpopulations in the repair phase of kidney IRI.
Figure S1. Kidney tissue levels of IL-2, IL-6 and IL-10 after Treg depletion at the 10 day-end point.
Detailed Methods
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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