Endogenous foxp3$^+$ T-regulatory cells suppress anti-glomerular basement membrane nephritis

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Foxp3$^+$ T-regulatory cells (Tregs) may suppress pathogenic inflammation; however, although transferred Tregs lessen glomerulonephritis in mice, the role of endogenous foxp3$^+$ cells is not known. To study this, we characterized endogenous foxp3$^+$ cells in accelerated anti-glomerular basement membrane (GBM) nephritis by using foxp3^{GFP} reporter mice to track their responses in early and established disease. Further, diphtheria toxin was used to ablate foxp3$^+$ Tregs in foxp3^{DTR} mice after establishing an immune response. In this model, mice were immunized with sheep globulin in adjuvant, and sheep anti-mouse GBM globulin was injected after 4 days to initiate progressive histological and functional injury. Intrarenal leukocytic infiltrates were increased by day 3 but intrarenal foxp3$^+$ Tregs, present in interstitial and periglomerular areas, were only increased at day 7. Ablation of foxp3$^+$ Tregs after injection of anti-GBM globulin increased renal injury and systemic T-cell responses, including increased interferon-γ and interleukin-17A (IL-17A) production, but no change in antibody titers. Compared with foxp3$^+$ Tregs isolated from naive mice, those from immunized mice produced more IL-10 and more effectively regulated CD4$^+$foxp3$^-$ responder T cells. Thus, endogenous foxp3$^+$ Tregs infiltrate the kidney in glomerulonephritis, and deleting foxp3$^+$ cells after the induction of immune responses upregulated T-cell reactions and enhanced disease. Hence, endogenous foxp3$^+$ cells have increased suppressive capacity after immune stimuli.

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T cells that express the transcription factor foxp3 are a regulatory subset of CD4$^+$ helper T cells, necessary for the maintenance of peripheral tolerance[1,2] and immune homeostasis.[3,4] Foxp3$^+$ T-regulatory cells (Tregs) are the most well-characterized subset of Tregs, but not all subsets of Tregs express foxp3.[5] Foxp3$^+$CD4$^+$ cells are reciprocally linked with the development of Th17 effector cells,[6,7] and recent studies suggest that these foxp3$^+$ Tregs cells can regulate both Th1-[8] and Th17-[9]mediated effector responses. In experimental murine glomerulonephritis, both Th1 and Th17 effector subsets, and their respective signature cytokines, interferon-γ (IFN-γ) and interleukin-17A (IL-17A), can mediate severe glomerular disease.[10–13]

In renal autoimmunity, regulation of autoreactive T-cell responses to the Goodpasture antigen by CD4$^+$CD25$^+$ T cells has been demonstrated in convalescent Goodpasture's disease patients.[14] In murine models of crescentic glomerulonephritis, transfer of exogenous naive CD4$^+$ CD25$^+$ T cells before induction of nephritis attenuates disease,[15] and affecting the migration of Tregs to secondary lymphoid organs[16] or the kidney[17] worsens injury. In murine models of toxic and ischemic renal injury, transfer of foxp3-transduced CD4$^+$ cells or naive CD25$^+$ cells, or anti-CD25 antibody administration, attenuates injury.[18–24]

Although studies suggest a role for foxp3$^+$ Tregs in suppressing renal disease, most have focused on cell transfer; therefore, any role for endogenous foxp3$^+$ cells in limiting glomerular disease directed by antigen-specific CD4$^+$ cells has not been demonstrated directly. This question can be addressed using foxp3^{GFP} reporter mice[25] and foxp3^{DTR} mice.[4] Foxp3^{GFP} mice[25] possess a green fluorescent protein (GFP)–foxp3 fusion protein-reporter knock-in allele that allows foxp3$^+$ cells to be identified and isolated for functional experiments. Foxp3^{DTR} mice[4] express the human diphtheria toxin (DT) receptor under the control of the *foxp3* locus, allowing specific ablation of this regulatory T-cell subset on administration of DT.

The aim of the current studies is to define the role of endogenous foxp3$^+$ Tregs in anti-glomerular basement membrane (GBM) nephritis.[26] The availability of foxp3^{DTR}

mice allows depletion of Tregs after initiating an immune response to the nephritogenic antigen (in this model, sheep globulin). Previously used strategies for depleting endogenous Tregs using anti-CD25 antibodies would, in this situation, deplete effector T cells as well.[27] The availability of foxp3^{DTR} mice, used in the current studies, overcomes this

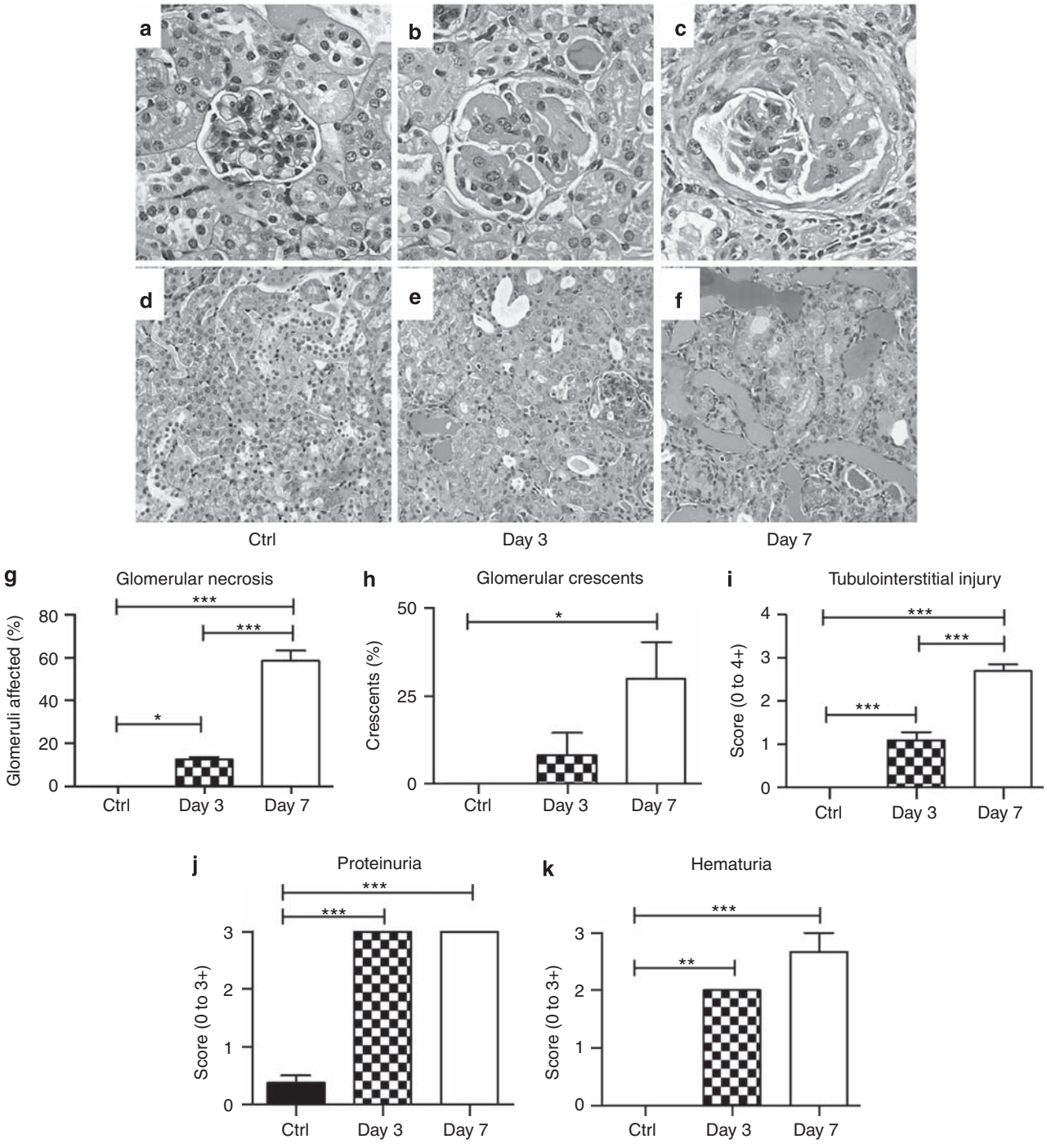

Figure 1 | Histological and functional renal injury at days 3 and 7 in accelerated anti-GBM nephritis using foxp3^{GFP} reporter mice. Accelerated anti-glomerular basement membrane (GBM) nephritis was induced in foxp3^{GFP} mice that were culled on day 3 or day 7. Control mice (ctrl) were immunized but did not receive sheep anti-GBM globulin. (**a**–**c**) Representative periodic acid-Schiff (PAS) stains of glomerular cross-sections at high power, ×400, showing no histological abnormalities in ctrl mice, mild glomerular necrosis in day 3 mice, and more severe necrosis with crescent formation in day 7 mice. (**d**–**f**) PAS staining of the tubulointerstitial compartment at low power, ×200, showing no tubulointerstitial injury in ctrl mice, mild tubular dilation with some cast formation present in day 3 mice, and tubular atrophy with markedly increased cast formation and cellular infiltrate in day 7 mice. Assessments of histological renal injury show (**g**) glomerular necrosis, (**h**) glomerular crescents, and (**i**) tubulointerstitial in ctrl, day 3, and day 7 mice. (**j**) Proteinuria and (**k**) hematuria levels, measured by urine test strips. *$P<0.05$, **$P<0.01$, and ***$P<0.001$ by analysis of variance, Tukey's post-test.

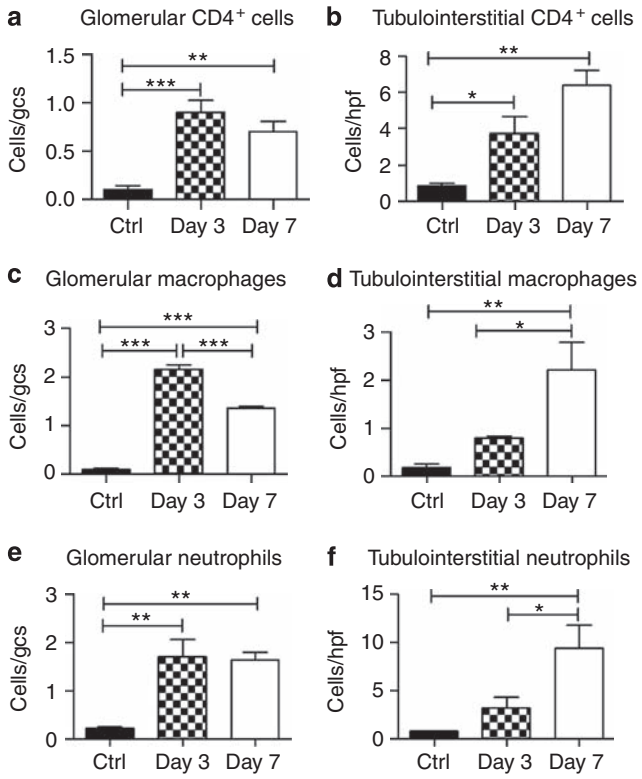

Figure 2 | Infiltration of cellular effectors into glomerular and tubulointerstitial compartments in early, day 3, and established, day 7, disease. Immunohistological staining on periodate lysine paraformaldehyde-fixed frozen kidney sections of renal (**a**, **b**) CD4$^+$ T cells, (**c**, **d**) macrophages, and (**e**, **f**) neutrophils in control (ctrl), day 3, and day 7 mice. *$P<0.05$, **$P<0.01$, ***$P<0.001$ by analysis of variance, Tukey's post-test. gcs, glomerular cross-section; hpf, high-power field.

problem by allowing specific deletion of Tregs on the basis of their lineage-specific expression of foxp3.

RESULTS

Endogenous foxp3$^+$ Tregs infiltrate the kidney by day 7, not day 3, of glomerulonephritis

Renal injury and infiltrates in this model of accelerated anti-GBM disease were characterized using foxp3^{GFP} reporter mice.[24] Mice were immunized with normal sheep globulin 4 days before intravenous injection of sheep anti-GBM globulin on day 0, and humanely killed on day 3 or 7. Control mice were immunized with sheep globulin, and killed on day 7. Mice injected with anti-GBM globulin developed focal and segmental necrosis, glomerular crescent formation, tubulointerstitial injury (tubular dilation and atrophy, sloughing of tubular epithelial cells and cast formation), proteinuria and hematuria (described in Figure 1). Immunochemical staining revealed glomerular CD4$^+$ T cells, macrophages, and neutrophils at day 3 but no further increase at day 7 (Figure 2a, c and e) and progressively increased numbers of tubulointerstitial CD4$^+$ cells, macrophages, and neutrophils (Figure 2b, d and f).

Immunohistological staining for foxp3 on paraffin-embedded kidney sections showed that foxp3$^+$ Tregs were rarely found in control or in day 3 mice but were readily detected in day 7 mice, predominantly in periglomerular and tubulointerstitial areas (Figure 3a–f and g). Foxp3$^+$ cells were rarely found in glomeruli. Using flow cytometry, endogenous foxp3^{GFP} numbers were increased at day 7 (compared with control and day 3 mice; Figure 3h). As a proportion of all intrarenal leukocytes (CD45$^+$ cells), foxp3$^+$ Tregs were increased at day 7 compared with control and day 3 mice: 1.9 versus 1.1 and 1.2%, respectively (Figure 3i). As a proportion of intrarenal CD4$^+$ T cells, compared with control, foxp3$^+$ Tregs were decreased at day 3, but increased by day 7: 11.0, 7.3, and 13.8%, respectively (Figure 3j). Representative flow cytometry plots of the intrarenal CD45$^+$ cells, gated on CD4$^+$ and foxp3^{GFP+} cells, are shown in Figure 3k. Dual immunofluorescence confocal microscopy of kidney sections stained with an antigen-presenting cell-conjugated anti-CD4$^+$ antibody (using endogenous GFP expressed by foxp3^{GFP} mice) confirmed the presence of intrarenal CD4$^+$foxp3$^+$ Tregs in kidneys of day 7 mice (Figure 4a–c).

Foxp3$^+$ Tregs in the draining inguinal lymph nodes enumerated by flow cytometry and expressed as a proportion of CD45$^+$ or CD4$^+$ cells were not different between control, day 3, and day 7 groups (Table 1). There was a trend toward a decrease in the total number of foxp3$^+$ Tregs and an increase in foxp3$^+$ Tregs as a proportion of CD45$^+$ cells. In this model of anti-GBM nephritis, the majority of foxp3$^+$ Tregs in the kidney and inguinal draining lymph nodes were CD25$^+$, whereas others were CD25$^-$ (Supplementary Figure S1 online). By day 7, a higher proportion of foxp3$^+$ cells in the kidney were CD25$^-$ compared with control mice.

Depletion of endogenous foxp3$^+$ Tregs after induction of anti-GBM nephritis exacerbates disease

Foxp3^{DTR} mice were studied to determine the role of endogenous foxp3$^+$ Tregs in the effector phase of disease. Mice were immunized with normal sheep globulin, then after 4 days injected with sheep anti-mouse GBM globulin. Foxp3$^+$ Tregs were ablated in foxp3^{DTR} mice by intraperitoneal 1 μg DT injections after anti-GBM globulin on the day of injection, with further injections after 24 and 72 h, according to previously published protocols.[4,28] This depletion strategy ensured that immune responses to sheep globulin had been established, with depletion occurring after the induction of anti-GBM nephritis. Control C57BL/6 wild-type (WT) mice with glomerulonephritis received equal doses of DT. WT mice developed moderate histological disease at day 10, characterized by glomerular focal and segmental necrosis, crescent formation, and tubulointerstitial injury. All disease parameters were exacerbated in foxp3^{DTR} mice after depleting foxp3$^+$ Tregs (Figure 5a–h).

Compared with WT mice, endogenous foxp3$^+$ depletion in foxp3^{DTR} mice resulted in an increase in CD4$^+$ T cells, macrophages, and neutrophils both in glomeruli and the tubulointerstitium (Figure 6a–f). The proportions and

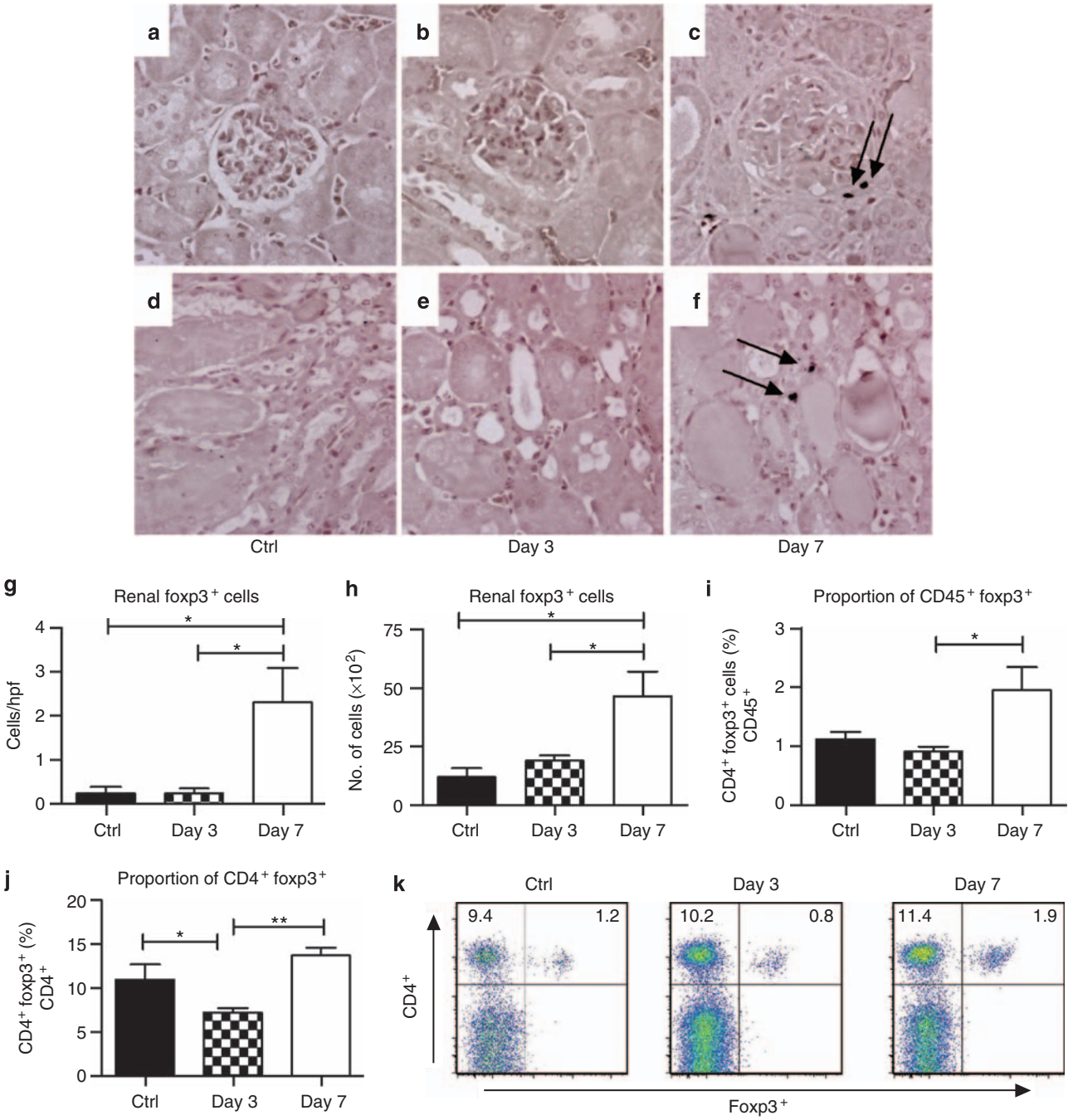

Figure 3 | Foxp3$^+$ Tregs have infiltrated the kidney only by day 7. Representative diaminobenzidine (black) staining for foxp3 on formalin-fixed kidney sections (**a**–**c**) around glomeruli and (**d**–**f**) within the tubulointerstitium, at high power × 400, in control (ctrl), day 3, and day 7 mice. Arrows show foxp3-positive staining. (**g**) Quantification of renal foxp3$^+$ T-regulatory cells (Tregs) by immunohistology. (**h**) Enumeration of foxp3$^+$ Tregs by flow cytometry in a whole kidney. (**i**) Foxp3$^+$ Tregs presented as a proportion of renal CD45$^+$ cells and (**j**) Foxp3$^+$ Tregs presented as a proportion of renal CD4$^+$ cells. (**k**) Illustrative FACS plot showing populations of CD4$^+$ cells on the *y* axis and foxp3$^+$ cells on the *x* axis (numbers within quadrants are the respective percentages) in ctrl, day 3, and day 7 mice. *$P<0.05$ and **$P<0.01$ by analysis of variance, Tukey's post-test. hpf, high-power field.

activation status of intrarenal CD4$^+$ T cells were analyzed by flow cytometry. As a proportion of CD45$^+$ cells, foxp3^{DTR} mice had increased CD4$^+$ cells (Figure 7a and b), but proportions of intrarenal CD4$^+$ cells expressing the effector memory phenotype (CD4$^+$CD44$^+$) were unchanged (Figure 7c and d).

Systemic immune responses are enhanced after foxp3$^+$ cell depletion in an established immune response

Supernatant from sheep globulin-stimulated splenocyte cultures was analyzed by cytometric bead array for IFN-γ, IL-17A, tumor necrosis factor, and IL-6. The prototypic Th1 cytokine IFN-γ or the Th17 cytokine IL-17A was not detected

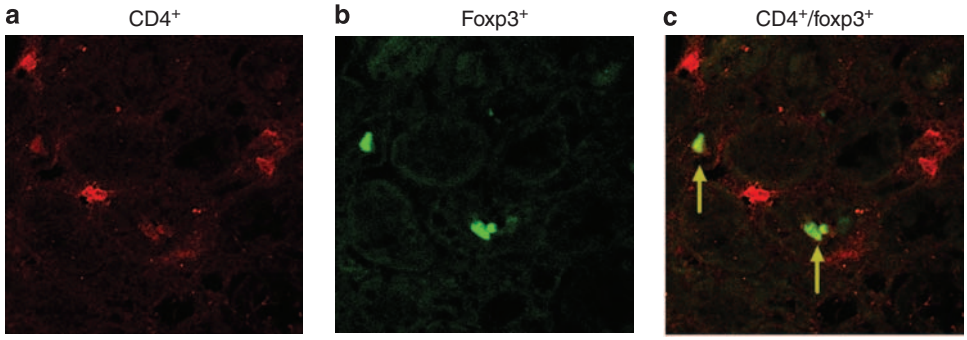

Figure 4 | Detection of intrarenal CD4$^+$foxp3$^-$ cells and CD4$^+$foxp3$^+$ T-regulatory cells using dual immunofluorescence confocal microscopy in established disease. Kidney sections from mice with established anti-glomerular basement membrane nephritis (day 7) were stained with anti-CD4 antibodies, and foxp3$^+$ cells were identified by green fluorescent protein expression. (**a**) CD4$^+$ cells (red). (**b**) Foxp3$^+$ cells (green). (**c**) Merge: double-positive cells (yellow arrows) are seen along with single-positive CD4$^+$foxp3$^-$ cells (red). Micrographs were taken at $\times$600.

Table 1 | Foxp3$^+$ T-regulatory cell numbers and proportions of CD45$^+$ and CD4$^+$ cells in the draining lymph node

	CD4$^+$foxp3$^+$ No. ($\times 10^3$)	CD4$^+$foxp3$^+$ CD45$^+$ (%)	CD4$^+$foxp3$^+$ CD4$^+$ (%)
Ctrl	28.3 ± 6.8	1.29 ± 0.1	5.65 ± 0.4
Day 3	30.2 ± 10.2	1.20 ± 0.1	5.40 ± 0.5
Day 7	13.3 ± 4.3	1.68 ± 0.2	5.40 ± 0.6

Abbreviations: Ctrl, control; Tregs, T-regulatory cells.
Total numbers of foxp3$^+$ Tregs were enumerated by flow cytometry from the two inguinal draining lymph nodes and expressed as proportions of CD45$^+$ as well as CD4$^+$ cells in control-immunized mice at day 3 or at day 7.

in splenocyte supernatants from WT mice (limit of assay: 20 pg/ml), but both were found in supernatants from all foxp3^{DTR} mice (Figure 8a and b). Tumor necrosis factor and IL-6 were also increased in foxp3^{DTR} mice (Figure 8c and d). Titers of mouse anti-sheep immunoglobulin G (IgG) antibodies were unchanged in foxp3^{DTR} mice (Figure 8e). CD4$^+$ effector T cells were assessed in lymph nodes by removing the draining inguinal lymph nodes at day 10, counting cell numbers, then analyzing the proportions of CD4$^+$CD44$^+$ effector memory T cells. Compared with WT mice, foxp3$^+$ cell-depleted mice had more total leukocytes in the draining nodes (Figure 9a). The proportion of CD4$^+$ T cells within the lymph node cell population was not different between groups, but foxp3$^+$ cell-depleted mice had a higher proportion of CD4$^+$ cells expressing CD44 (Figure 9b–d).

Foxp3$^+$ Tregs from mice immunized with sheep globulin have increased immunosuppressive capacity

To determine whether endogenous foxp3$^+$ Tregs have an increased suppressive capacity following immune stimulation, the potency of 'activated' foxp3$^+$ Tregs (from immunized mice) in suppressing effector T-cell responses was compared with that of naive foxp3$^+$ Tregs. Responder CD4$^+$foxp3$^-$ T cells from sheep globulin-immunized mice were cultured with either CD4$^+$foxp3$^+$ Tregs from naive mice or with CD4$^+$foxp3$^+$ Tregs from mice immunized with sheep globulin. Different foxp3$^+$ Treg/responder T-cell ratios were used, with mitomycin C-treated, CD4-depleted naive splenocytes, and sheep IgG included in cultures. Compared with foxp3$^+$ Tregs from naive mice, foxp3$^+$ Tregs from immunized mice had a greater capacity to inhibit proliferation of CD4$^+$foxp3$^-$ responder T cells (Figure 10a). IFN-γ secretion from CD4$^+$foxp3$^-$ responder T cells was suppressed below the assay's detection limit (20 pg/ml) when cocultured with foxp3$^+$ Tregs from immunized mice, but was detectable at a ratio of 1:16 and 1:8 when mixed with foxp3$^+$ Tregs from naive mice (Figure 10b). Tregs from immunized mice were also more potent inhibitors of IL-17A (Figure 10c). IL-2 and tumor necrosis factor secretion levels were also markedly reduced by foxp3$^+$ Tregs and more effectively with Tregs from immunized mice. Tregs (2×10^4 cells) from immunized mice secreted more IL-10 when cultured without responder T cells compared with foxp3$^+$ Tregs from naive mice (Figure 10f). At a ratio of 1:2 (foxp3$^+$ Treg (1×10^4 cells) to responder T cells (2×10^4 cells)), IL-10 was still detectable in immunized mice Tregs, but only marginally detectable in naive Treg cocultures.

DISCUSSION

The role of endogenous Tregs in nephritis has not yet been defined. The current studies characterize the evolution of a T-regulatory cell response by endogenous foxp3$^+$ Tregs during early inflammatory injury, at day 3, and when disease is established, at day 7, and demonstrates that depleting endogenous foxp3$^+$ Tregs after the induction of immune responses enhances T-cell effector responses and leads to more severe glomerulonephritis. Furthermore, following immunization, foxp3$^+$ Tregs have an increased ability to regulate helper T effector-mediated responses.

In this model of anti-GBM nephritis, mice were immunized with sheep globulin to induce an immune response against this antigen, and renal disease was triggered using sheep anti-mouse GBM globulin. In a similar model of nephritis, nephritic kidneys exhibited increased foxp3$^+$ Tregs at day 7.[17] However, whether these endogenous foxp3$^+$ Tregs

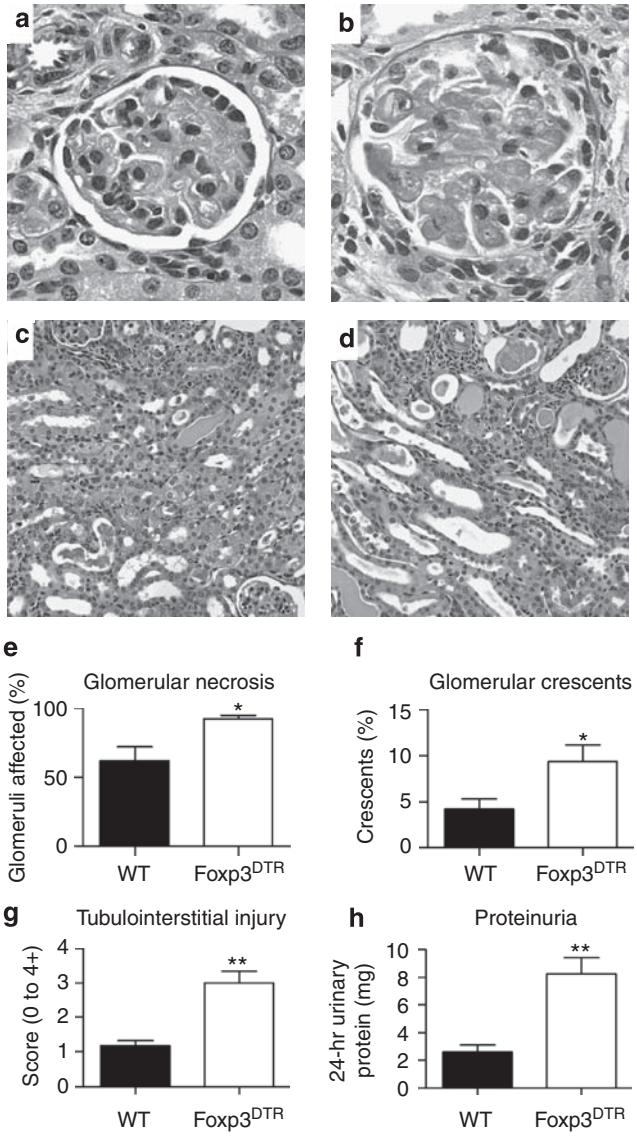

Figure 5 | Specific ablation of foxp3$^+$ T-regulatory cells after the induction of disease enhances anti-glomerular basement membrane nephritis. Foxp3$^+$ T-regulatory cells were ablated in foxp3^{DTR} mice following injection of anti-glomerular basement membrane globulin and humanely killed on day 10. Disease outcome was compared with WT mice. Representative photomicrographs of periodic acid-Schiff (PAS)-stained sections at high power ($\times$400) in (**a**) WT mice showing some segmental necrosis and (**b**) Foxp3^{DTR} mice showing more severe necrosis and early crescent formation. PAS-stained sections showing the tubulointerstitium at lower power ($\times$200) in (**c**) WT mice showing mild tubular dilation and some cast formation, and (**d**) foxp3^{DTR} mice showing more severe tubular dilation, tubular atrophy, and more cast formation. PAS-stained kidney sections were assessed for (**e**) glomerular necrosis, (**f**) glomerular crescents, and (**g**) tubulointerstitial injury. (**h**) Proteinuria was assessed on a 24-h collection before the end of the experiment. *$P<0.05$ and **$P<0.01$ by Student's *t*-test. WT, wild type.

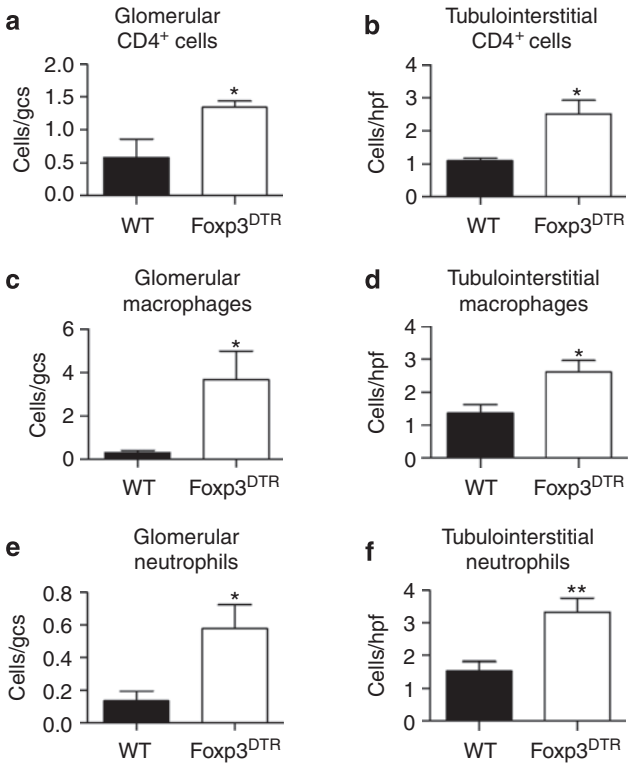

Figure 6 | Renal cellular effectors are increased following ablation of foxp3$^+$ T-regulatory cells. Kidney sections from WT and foxp3^{DTR} mice were stained to assess the infiltration of cellular effectors within the glomerular and tubulointerstitial areas: (**a**, **b**) CD4$^+$ T cells, (**c**, **d**) macrophages, and (**e**, **f**) neutrophils. *$P<0.05$ and **$P<0.01$ by Student's *t*-test. gcs, glomerular cross-section; hpf, high-power field; WT, wild type.

were recruited into the kidney before or after effector cell infiltrates was not known. Tracking of foxp3$^+$ Tregs at days 3 and 7 within the kidney by immunohistology and flow cytometry showed that intrarenal foxp3$^+$ Tregs are increased in the kidney only at day 7, but not at day 3. As a proportion of CD4$^+$ cells, foxp3$^+$ Tregs are decreased at day 3, consistent with the influx of CD4$^+$foxp3$^-$ effector helper T cells into the kidney. Confocal microscopy confirmed that foxp3$^+$CD4$^+$ cells were readily found in the kidney at day 7. Therefore, tracking of endogenous renal foxp3$^+$ Tregs in the early and established phase of disease suggests that intrarenal foxp3$^+$ Tregs are unlikely to be important at the onset of nephritis but could be relevant after nephritic insult by cellular effectors has occurred. In the draining lymph nodes, total numbers of foxp3$^+$ Tregs and foxp3$^+$ Treg percentages as proportions of CD45$^+$ or CD4$^+$ were not significantly different between control, day 3, and day 7 mice, but there was a trend to decreased numbers of foxp3$^+$ Tregs at day 7, which may reflect emigration of Tregs from the node.

Before the development of foxp3^{DTR} mice, studies in other models of disease have used anti-CD25 antibodies to deplete Tregs.[29] This method does not deplete CD25$^-$ foxp3$^+$ cells,[2,25] and, in the context of an active immune response, may deplete activated T cells.[27] It is likely that depleting foxp3$^+$ cells before a nephritogenic immune response was established would enhance the subsequent adaptive immune response and therefore injury. However, as using foxp3^{DTR} mice means that Tregs can be depleted specifically, we sought

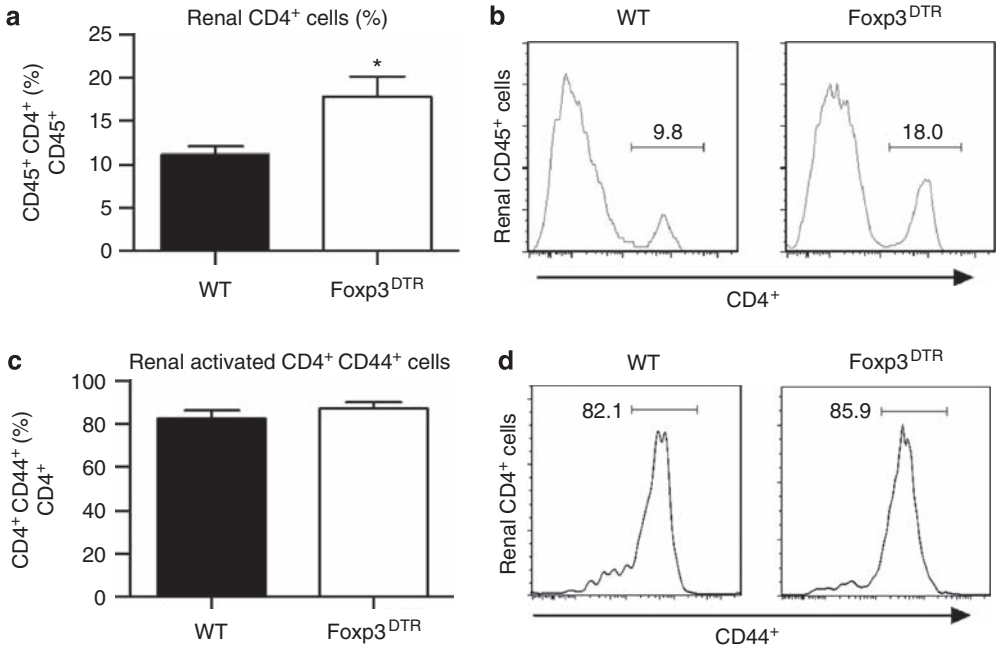

Figure 7 | Renal CD4$^+$ cell proportion is increased by flow cytometry. Kidneys of WT and foxp3^{DTR} mice were digested, and single cell suspensions were analyzed for CD45$^+$, CD4$^+$, and CD44$^+$ expression by flow cytometry. (**a**) Analysis of renal CD4$^+$ cells as a proportion of CD45$^+$ cells. (**b**) Illustrative FACS histograms showing a marked increased in the proportion of CD4$^+$ cells in the kidney (numbers are the percentages of CD4$^+$ cells). (**c**) Analysis of the proportion of CD4$^+$ cells that are of effector memory, CD4$^+$CD44$^+$, phenotype. (**d**) Illustrative FACS histograms of proportions of renal CD4$^+$ cells expressing CD44. *$P<0.05$ by Student's *t*-test. WT, wild type.

to address the role of endogenous foxp3$^+$ cells in the context of an active immune response. In the current studies, foxp3$^+$ Treg ablation after inducing nephritogenic immune responses led to more severe glomerulonephritis. Although there was also an increase in renal CD4$^+$ T cells as a proportion of CD45$^+$ cells, the proportion of CD4$^+$ T cells that were CD4$^+$CD44$^+$ effector memory phenotype was similar, showing that endogenous foxp3$^+$ Tregs limited recruitment to the kidney of both CD44$^+$ and CD44$^-$ CD4$^+$ cells.

Systemic immunity to the nephritogenic antigen, measured by splenocyte cytokine production, showed that ablation of endogenous foxp3$^+$ Tregs resulted in increased Th1 and Th17 cytokines (IFN-γ and IL-17A), as well as in increased proinflammatory cytokines tumor necrosis factor and IL-6. The increase in both IFN-γ and IL-17A suggests that, in this model, foxp3$^+$ Tregs regulate both Th1 and Th17 effector subsets. In the draining lymph node, foxp3^{DTR} mice had increased total number of cells and a marked increase in the proportions of CD4$^+$ T cells that were CD4$^+$CD44$^+$ effector memory T cells, showing that foxp3$^+$ Tregs regulate T-cell activation in secondary lymphoid organs in this model.[16] The timing of the depletion of foxp3$^+$ cells, after the induction of immune responses, allowed the development of intact humoral immunity, as serum anti-sheep IgG levels were unchanged.

The near absence of Tregs in glomeruli of mice with glomerulonephritis in this model, together with the markedly increased systemic T-cell response, implies, at least in the glomerular lesion, that Tregs exert their effects predominantly within secondary lymphoid organs. However, the presence of intrarenal Tregs within the tubulointerstitium raises the possibility of an additional local role for Tregs in regulating the tubulointerstitial component of this disease.

The availability of foxp3^{GFP} reporter allows for Tregs to be isolated from an activated immune system and used in functional assays without compromising viability. Reliably isolating regulatory T cells for functional assays was previously possible only in naive mice, as selecting Tregs on the basis of CD25 expression meant that in active immune responses effector CD4$^+$ cells expressing CD25 would be included as 'regulatory' cells.[27] In our *ex vivo* coculture experiments, foxp3$^+$ Tregs from immunized mice were better at suppressing T-cell effector functions (proliferation and secretion of proinflammatory cytokines) than were Tregs from naive mice. This increased suppressive effect of foxp3$^+$ Tregs from immunized mice suggests that endogenous Tregs upregulate their suppressive function in response to immune stimuli. Tregs from immunized mice secreted more IL-10, consistent with studies showing that this anti-inflammatory cytokine regulates disease in this model.[30] The effects of foxp3$^+$ Tregs from naive mice when cocultured at higher ratios of Tregs to T-responder cells are consistent with those of previous experiments demonstrating that transfer of exogenous CD4$^+$CD25$^+$ Tregs before inducing an immune response results in suppression of nephritis.[15]

In conclusion, the current studies characterize the infiltration of endogenous foxp3$^+$ Tregs into the kidney,

demonstrate their necessity in attenuating glomerulonephritis, and show that foxp3+ Tregs upregulate their regulatory capacity in nephritogenic inflammation.

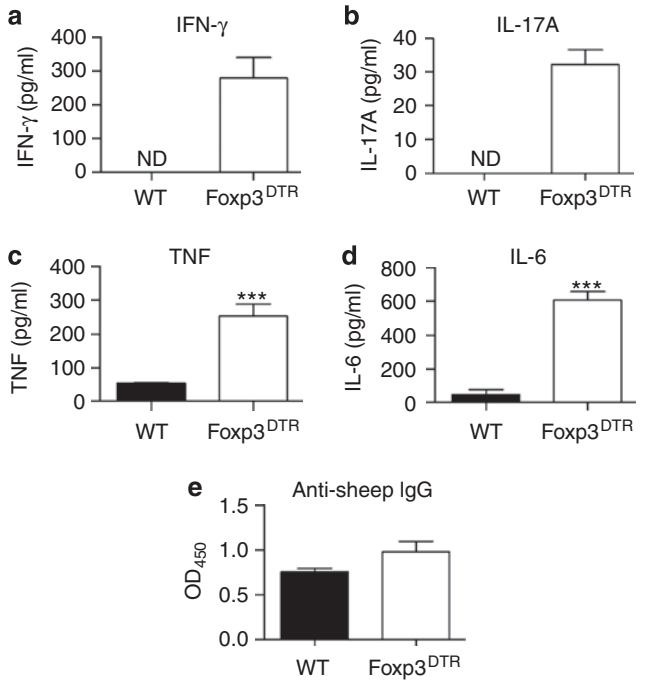

Figure 8 | Systemic cell-mediated immune responses, but not humoral responses, are increased following ablation of foxp3+ T-regulatory cells. Cytokines: (**a**) IFN-γ, (**b**) IL-17A, (**c**) TNF, and (**d**) IL-6 were measured by cytometric bead array from supernatants of cultured splenocytes from WT or foxp3DTR stimulated with sheep globulin at the end of the experiment. (**e**) Circulating anti-sheep IgG levels were measured by enzyme-linked immunosorbent assay (1:800 dilution). ***$P<0.001$ by Student's *t*-test. IFN-γ, interferon-γ; IgG, immunoglobulin G; IL, interleukin; ND, not detected; TNF, tumor necrosis factor; WT, wild type.

MATERIALS AND METHODS

Experimental design

Foxp3GFP female mice ($n=4$ per group) were housed at Monash Animal Services (Melbourne, Australia). Age- and sex-matched foxp3DTR mice ($n=6$), and control C56BL/6J mice ($n=5$) were housed at the Institute for Molecular Medicine and Experimental Immunology, Bonn, Germany. Accelerated anti-GBM glomerulonephritis was induced by immunizing mice subcutaneously with 0.5 mg (100 µl) of sheep globulin in Freund's complete adjuvant (100 µl) at day −4, followed by intravenous injection of sheep anti-mouse GBM globulin (0.15 or 0.20 mg/g) at day 0. Mice were humanely killed at day 3, 7, or 10. Foxp3+ Tregs were specifically ablated by injection of DT (Merck, Darmstadt, Germany) at days 0, 1, and 3. Control C57BL/6 mice with glomerulonephritis received equal doses of DT at the same time points. This method of foxp3+ cell depletion achieved an efficiency of ~90% when measured by flow cytometry at day 4 in the kidney, inguinal draining lymph nodes, and spleen (Supplementary Figure S2 online). Animal studies were conducted under specific pathogen-free conditions and approved by the Monash University Animal Ethics Committee or the German Institutional and Government Review Boards. Results are expressed as means ± s.e.m. Statistical analyses were conducted using analysis of variance when comparing three groups, followed by Tukey's post-test or Student's unpaired *t*-test when comparing two groups, *$P<0.05$, **$P<0.01$, ***$P<0.001$ (GraphPad Prism; GraphPad Software, San Diego, CA).

Assessment of renal injury and immune cell infiltration

Glomerular necrosis and crescents and tubulointerstitial injury were assessed on 3 µm-thick, periodic acid-Schiff-stained, Bouin's-fixed, paraffin-embedded sections, ⩾50 glomeruli per mouse. Glomerular necrosis was defined as accumulation of periodic acid-Schiff-positive material in ⩾50% of the glomerulus, and glomerular crescents as two or more layers of cells in Bowman's space. Tubulointerstitial injury was assessed in 15 randomly selected cortical areas from each animal at ×250 magnification using a 10 mm^2 graticule, and defined as tubular dilation, tubular atrophy, sloughing of tubular epithelial cells, and cast formation. Injury was

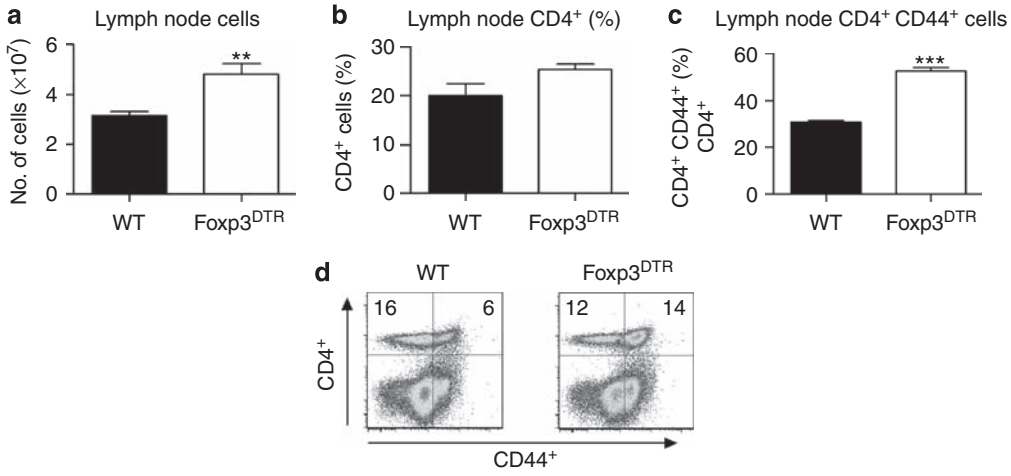

Figure 9 | CD4+ T-cell differentiation into CD4+CD44+ effector memory cells is upregulated in the draining inguinal lymph node following ablation of foxp3+ T-regulatory cells. Single cell suspensions from the draining inguinal lymph nodes were enumerated, stained for CD4+ and CD44+ expression, and analyzed by flow cytometry. (**a**) Total numbers of leukocytes from the two inguinal nodes. (**b**) Percentage of leukocytes expressing CD4+. (**c**) Percentage of CD4+CD44+ cells as a proportion of CD4+ cells. (**d**) Illustrative FACS plot showing a marked increase in the percentages of CD44-expressing CD4+ cells in foxp3DTR mice. **$P<0.01$ and ***$P<0.001$ by Student's *t*-test. WT, wild type.

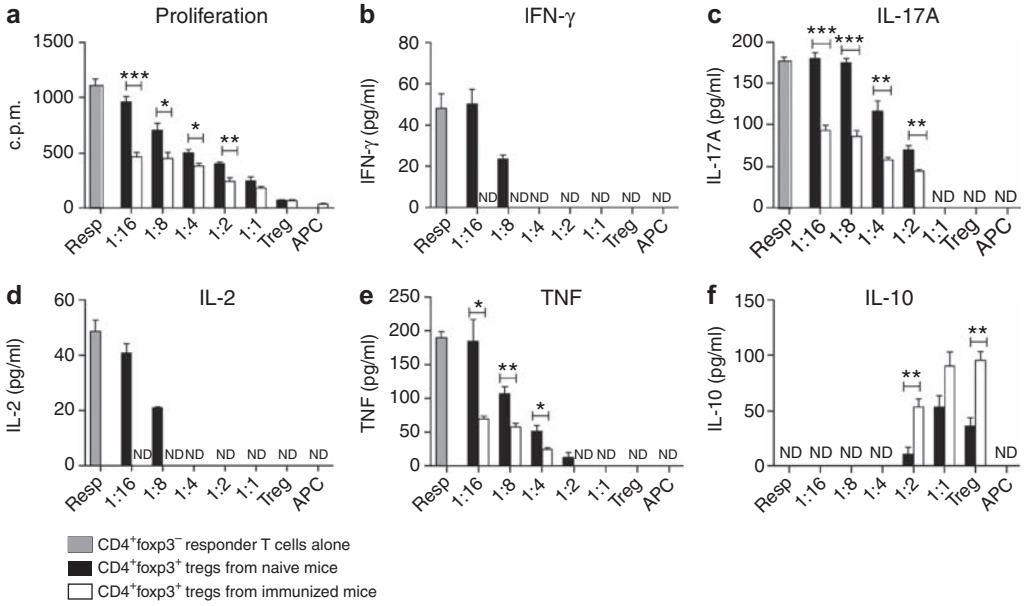

Figure 10 | Compared with foxp3$^+$ Tregs from naive mice, foxp3$^+$ Tregs from sheep globulin-immunized mice better suppress cell-mediated responses and secrete more IL-10 *ex vivo*. Foxp3$^+$ T-regulatory cells (Tregs) from lymph nodes were isolated either from mice immunized with sheep globulin or from naive mice, and their capacity to inhibit cell-mediated responses from CD4$^+$foxp3$^-$ responder T cells measured using a coculture assay. Responder T cells were cultured at a concentration of 2×10^4 cells per well with increasing ratios of foxp3$^+$ Tregs (1:16 to 1:1) in the presence of antigen-presenting cells and stimulating antigen sheep globulin. (**a**) [^{3}H]-thymidine proliferation, and secretion of proinflammatory cytokines (**b**) IFN-γ, (**c**) IL-17A, (**d**) IL-2, and (**e**) TNF, as well as secretion of anti-inflammatory (**f**) IL-10 from Tregs. Inhibition of responses from responder T cells and IL-10 secretion from foxp3$^+$ cells were compared. APC, antigen-presenting cells without foxp3$^+$ Tregs or responder T cells; IFN-γ, interferon-γ; IL, interleukin; ND, not detected; TNF, tumor necrosis factor; Treg, 2×10^4 foxp3$^+$ Tregs without responder T cells. *$P<0.05$, **$P<0.01$, and ***$P<0.001$ by Student's *t*-test.

graded according to a scale of 0–4: grade 0, 0%, grade 1, 0–25%; grade 2, 25–50%, grade 3; 50–75%; and grade 4, 75–100% of the tubulointerstitium injured. Urine was collected either by bladder puncture at the time of death or by metabolic cage 24 h before death. Proteinuria and hematuria was measured by Combur-Test strips (Roche Diagnostics, Castle Hill, Australia) or proteinuria by modified Bradford's method as previously described.[31]

CD4$^+$ T cells, macrophages, and neutrophils were shown by immunoperoxidase staining of 6 μm-thick, periodate lysine paraformaldehyde-fixed, frozen kidney sections as previously described.[32] Foxp3 was detected on formalin-fixed paraffin-embedded 3 μm sections. The primary monoclonal antibodies used were GK1.5 (anti-mouse CD4; American Type Culture Collection, Manassas, VA), FA/11 (macrophages, anti-mouse CD68; from Dr Gordon L. Koch, Cambridge, England), RB6–8C5 (neutrophils, anti-Gr-1), and anti-mouse foxp3 (FJK-16s; eBiosciences, San Diego, CA), with rabbit anti-rat biotin as a secondary antibody (BD Biosciences, North Ryde, Australia). To enumerate positive-stained cells, $\geqslant$20 consecutively viewed glomeruli and $\geqslant$15 high-power cortical interstitial fields (excluding perivascular regions) were assessed per animal as described previously.[32] Confocal images were captured using a NikonC1 inverted confocal laser and Nikon Ti-E scanning microscope from 6 μm SNAP frozen sections, blocked with 10% rat serum in 5% bovine serum albumin/phosphate buffered saline before incubating with antigen-presenting cell-conjugated CD4 antibodies (eBioscience).

Isolation of leukocytes and flow cytometry

Kidneys were digested with 1 mg/ml collagenase D (Roche Diagnostics) and 0.1 mg/ml DNAse I (Roche Diagnostics) in RPMI 1640/10% fetal calf serum (Invitrogen, Mount Waverley, Australia) and 10 mmol/l HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid). Spleens and lymph nodes were minced and filtered, erythrocytes lysed with ammonium chloride, and viability assessed by trypan blue staining. Single cell suspensions were stained with antibodies against CD4, CD25, CD44, and CD45 (all BD Biosciences). Foxp3$^+$ cells were assessed by endogenous GFP expression. Propidium iodide or Hoechst-positive cells were excluded from analyses. Total cells were enumerated with BD Calibrite Beads (BD Biosciences). Cell data were acquired on FACSCanto flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star, Ashland, OR).

Measurement of systemic immune responses

Cytokines were measured from supernatants of cultured splenocytes using single cell suspensions of splenocytes from each mouse. Splenocytes were cultured for 72 h (24-well tissue culture plates, 1 ml aliquots, 4×10^6cells/ml, RPMI-1640, 10% fetal calf serum, 1% penicillin/streptomycin, 2 mmol/l L-glutamine, 50 μmol/l 2-mercaptoethanol), with 10 μg/ml protein G-purified sheep IgG, as previously described[32,33] using a BD Cytometric Bead Array Mouse Th1/Th2/Th17 Cytokine kit according to the manufacturer's instructions (BD Biosciences). Spleens from each individual mouse in both groups were assessed for proportions of CD4$^+$ cells and CD4$^+$foxp3$^+$ cells. The proportions of CD4$^+$ cells and CD4$^+$foxp3$^+$ Tregs in the spleen were similar between WT and foxp3^{DTR} mice at day 10 (CD4$^+$ WT 16.2 ± 3.0, foxp3^{DTR} 14.9 ± 1.7, of CD45$^+$ splenocytes; CD4$^+$foxp3$^+$ WT 3.5 ± 0.8, foxp3^{DTR} 3.5 ± 0.3, of CD4$^+$ cells), consistent with previous published data showing the return of CD4$^+$foxp3$^+$ Tregs to normal levels after

5–10 days after DT injections.[4] In separate experiments, cytokines were measured from cocultures of foxp3$^+$ Treg cells (from immunized or naive foxp3^{GFP} mice) with foxp3$^-$ responder cells at the ratios indicated. Circulating mouse anti-sheep IgG levels were assessed from serum as described previously[33] using horseradish peroxidase-conjugated sheep anti-mouse IgG (1:800 dilution, Amersham Biosciences, Rydalmere, Australia).

Coculture of CD4$^+$foxp3$^+$ Tregs with CD4$^+$foxp3$^-$ responder cells

CD4$^+$ cells were isolated by magnetic cell sorting according to the manufacturer's instructions (MACS CD4$^+$ T cell Isolation Kit; Miltenyi Biotec, North Ryde, Australia), followed by sorting based on GFP expression using cell sorter, Beckman Coulter MoFlo XDP (Beckman Coulter, Gladesville, Australia). CD4$^+$foxp3$^-$ responder T cells (2×10^4) were cocultured with increasing amounts of CD4$^+$foxp3$^+$ Tregs (1.25×10^3–2×10^4) and with erythrocyte-lysed, MACS CD4-depleted, mitomycin C-treated (50 μg/ml, 30 min, 37 °C, then washed repeatedly) splenocytes (8×10^4) and 100 μg/ml sheep IgG in 96-well round-bottom plates for 72 h. [^{3}H]-thymidine (0.5 μCi) was added per well for the last 16 h of culture and proliferation measured as counts per million, using a liquid scintillation β-counter (Cambridge Scientific, Cambridge, MA).

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Figure 1. Foxp3$^+$ Tregs in the kidney and in the inguinal draining lymph node are either CD25$^-$ or CD25$^+$.
Figure 2. Depletion efficiency of foxp3$^+$ Tregs in foxp3^{DTR} mice at day 4.
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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