

Regulatory T cells control the Th1 immune response in murine crescentic glomerulonephritis

Hans-Joachim Paust^{1,6}, Annett Ostmann^{2,6}, Annette Erhardt², Jan-Eric Turner¹, Joachim Velden³, Hans-Willi Mittrücker⁴, Tim Sparwasser⁵, Ulf Panzer¹ and Gisa Tiegs²

¹III Medizinische Klinik, Universitätsklinikum Hamburg-Eppendorf, Hamburg, Germany; ²Institut für Experimentelle Immunologie und Hepatologie, Universitätsklinikum Hamburg-Eppendorf, Hamburg, Germany; ³Institut für Pathologie, Universitätsklinikum Hamburg-Eppendorf, Hamburg, Germany; ⁴Institut für Immunologie, Universitätsklinikum Hamburg-Eppendorf, Hamburg, Germany and ⁵Institut für Infektionsimmunologie, Medizinische Hochschule Hannover, Hannover, Germany

Crescentic glomerulonephritis is mediated by inappropriate humoral and cellular immune responses toward self-antigens that may result from defects in central and peripheral tolerance. Evidence now suggests that regulatory T cells (Tregs) may be of pathophysiological importance in proliferative and crescentic forms of glomerulonephritis. To analyze the role of endogenous Tregs in a T cell-dependent glomerulonephritis model of nephrotoxic nephritis, we used 'depletion of regulatory T cell' (DEREG) mice that express the diphtheria toxin receptor under control of the FoxP3 (forkhead box P3) gene promoter. Toxin injection into these mice efficiently depleted renal and splenic FoxP3⁺ Treg cells as determined by fluorescent-activated cell sorting (FACS) and immunohistochemical analyses. Treg depletion exacerbated systemic and renal interferon- γ (IFN γ) expression and increased recruitment of IFN γ -producing Th1 cells into the kidney without an effect on the Th17 immune response. The enhanced Th1 response, following Treg cell depletion, was associated with an aggravated course of glomerulonephritis as measured by glomerular crescent formation. Thus, our results establish the functional importance of endogenous Tregs in the control of a significantly enhanced systemic and renal Th1 immune response in experimental glomerulonephritis.

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Crescentic glomerulonephritis is mediated by inappropriate humoral and cellular immune responses toward not well-characterized (self) antigens that might result from defects in central and peripheral tolerance. CD4⁺CD25⁺FoxP3⁺ regulatory T cells (Tregs) are generally thought to be of critical importance for the maintenance of peripheral tolerance.¹ Until now, little is known about the suppressive mechanisms mediated by CD4⁺CD25⁺FoxP3⁺ Tregs and their behavior during proliferative and crescentic glomerulonephritis.^{2,3} With respect to human disease, one report indicates that CD25⁺ T cells emerge following acute episodes of Goodpasture's disease suppressing the response to the Goodpasture autoantigen.⁴ Furthermore, the potential of Tregs to suppress the pathogenic immune responses in kidney disease has recently been proven in a model of crescentic glomerulonephritis in mice. The adoptive transfer of CD4⁺CD25⁺ Tregs showed that Tregs downregulate the nephritogenic immune response directly at the systemic site of antigen-specific T-cell priming, namely the renal lymph node.⁵ In consecutive studies, Eller *et al.*⁶ demonstrated that the chemokine receptor CCR7 (chemokine (C-C motif) receptor 7) regulates the trafficking of Tregs to the lymph node in experimental glomerulonephritis, which is essential for their anti-inflammatory properties. Our group recently provided evidence that Treg cells migrate in a CCR6–CCL20-dependent manner into the inflamed kidney in crescentic glomerulonephritis and might locally suppress immune-mediated renal inflammation.⁷

This study was designed to examine the potential role of endogenous Tregs in crescentic glomerulonephritis. We therefore induced the T cell-dependent model of nephrotoxic nephritis (NTN) in 'depletion of regulatory T cell' (DEREG) mice that express the diphtheria toxin (DTx) receptor and green fluorescent protein (GFP) under control of the FoxP3 (forkhead box P3) promoter, allowing selective and efficient depletion of FoxP3⁺ Treg cells by DTx injection.⁸ Hence, we intended to address whether Treg depletion influences the clinical course of experimental glomerulonephritis, and if so, to study their potential mechanisms of action.

Correspondence: Gisa Tiegs, Universitätsklinikum Hamburg-Eppendorf, Martinistrasse 52, 20246 Hamburg, Germany. E-mail: g.tiegs@uke.de

⁶These authors contributed equally to this work.

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RESULTS

Functional involvement of endogenous Tregs in NTN

To investigate the role of endogenous Tregs, we determined the frequency of CD4⁺CD25⁺FoxP3⁺ Tregs upon induction of NTN in a time course experiment. Interestingly, the number of Tregs was increased from day 5 onward, as shown by immunohistological FoxP3 staining (Figure 1a and b).

To evaluate the immunosuppressive capacity of Tregs from nephritic mice compared with their counterparts from healthy control mice, we isolated splenic CD4⁺CD25⁺ Tregs from both nephritic and healthy control mice and performed T-cell co-culture experiments. Enzyme-linked immunosorbent assay (ELISA) analysis of supernatants from co-cultures of Tregs and CD4⁺CD25⁻ responder T cells indicated that Tregs from nephritic mice maintained their *in vitro* suppressive capacity and might be even more potent than Tregs from healthy controls to inhibit the production of interleukin (IL)-2 and interferon- γ (IFN γ) by responder T cells (Figure 1c, IL-2 (pg/ml): single culture of responder T cells: 334.3 \pm 22.0; responder T cells + Tregs (control): 79.3 \pm 10.5; responder T cells + Tregs (NTN): 43.7 \pm 13.5; ****P* < 0.0001; IFN γ (pg/ml): single culture of responder T cells: 8490 \pm 772, responder T cells + Tregs (control): 6084 \pm 2949, responder T cells + Tregs (NTN): 4137 \pm 845.2; **P* < 0.05).

Depletion of Tregs in DREG mice by injection of DTx

To examine the involvement of endogenous Tregs in the model of crescentic glomerulonephritis, we used C57BL/6

DEREG mice in which specific depletion of FoxP3⁺ Tregs by injection of DTx and tracking of green fluorescent Tregs by fluorescent-activated cell sorting (FACS) analysis can be performed. To assess the efficiency of Treg depletion after repetitive injection of 1 μ g DTx per mouse (day -1 and day 3 upon NTN induction), FoxP3 expression was quantified in spleen and kidney at 7 days after NTN induction. Indeed, frequency of CD4⁺FoxP3⁺ Tregs was significantly reduced in both spleen (Figure 2a; 7.8 vs <1%) and kidney tissue (Figure 2b; 20.1 vs 2.6%) of DTx-treated mice in contrast to control nephritic mice as shown by intracellular FoxP3 staining with subsequent FACS analysis. GFP expression was also reduced in accordance to FoxP3 expression (data not shown). Successful renal Treg depletion was confirmed by histological staining of FoxP3 (Figure 2c). The frequency of Tregs was significantly reduced by ~80% at 4 days after the second DTx challenge (Figure 2d: FoxP3⁺ Tregs (cells per low power field): 24.7 \pm 2.7 vs 5.3 \pm 1.5; ***P* < 0.01).

Aggravated NTN in Treg-depleted nephritic mice

Next, we induced NTN in C57BL/6 wild-type (wt) and DREG mice with and without Treg depletion. Examination of periodic acid-Schiff (PAS)-stained kidney sections in the T cell-mediated autologous phase at day 7 after injection of the nephritogenic sheep serum revealed glomerular and tubulointerstitial damage. First, PAS-positive material was deposited intraglomerularly. Moreover, formation of cellular crescents and hypercellularity account for glomerular

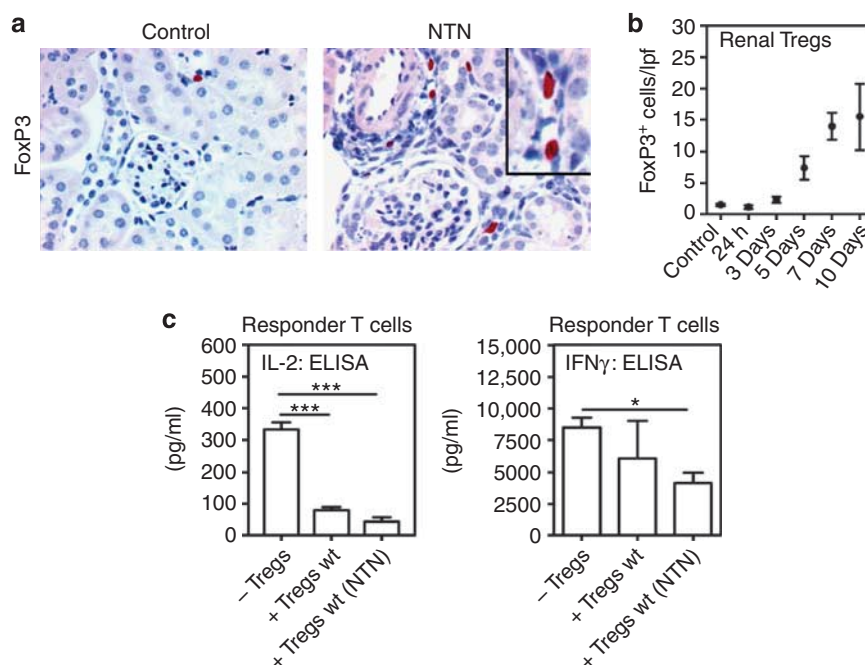


Figure 1 | Endogenous regulatory T cells (Tregs) in the murine model of nephrotoxic nephritis (NTN). (a) Representative immunohistochemistry for Tregs (forkhead box P3 (FoxP3)) in kidney sections from nephritic mice and nonnephritic controls (original magnification \times 400). (b) Quantification of tubulointerstitial FoxP3⁺ T-cell infiltration per low power field (lpf; original magnification \times 100) during the time course of NTN (*n* \geq 4). (c) 1×10^5 Splenic CD4⁺CD25⁻ responder T cells were cultured without or with 1×10^5 splenic CD4⁺CD25⁺ Tregs from nonnephritic wild-type (wt) controls or nephritic wt mice and stimulated with plate-bound anti-CD3 monoclonal antibody (mAb) for 72 h. Secretion of the cytokines interleukin-2 (IL-2) and interferon- γ (IFN γ) was assessed in supernatants by enzyme-linked immunosorbent assay (ELISA; **P* < 0.05, ****P* < 0.0001).

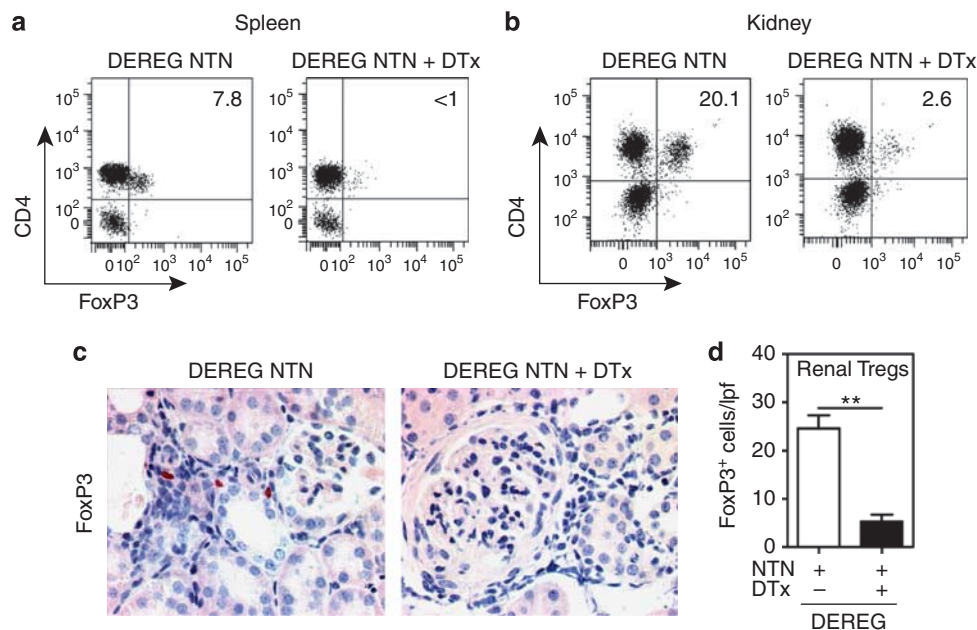


Figure 2 | Depletion of regulatory T cells (Tregs) in DEREg (depletion of regulatory T cell) mice by diphtheria toxin (DTx). Systemic depletion of Tregs was monitored by fluorescent-activated cell sorting (FACS) analysis of (a) splenic and (b) renal CD4⁺ T cells isolated from DEREg mice 7 days after induction of nephrotoxic nephritis (NTN) and 4 days after second application of DTx. (c) Representative kidney sections from nephritic DEREg mice without and with DTx treatments show FoxP3⁺ staining. (d) Quantification of renal and splenic FoxP3⁺ T cells indicates a strong reduction in numbers of FoxP3⁺ T cells after DTx treatments (***P* < 0.01).

alterations (Figure 3a). In the tubulointerstitial compartment, aberrant leukocyte infiltration and focal destruction of the regular tissue structure were detectable indicated by tubular dilatation and intratubular protein casts (data not shown).

To quantify glomerular and tubular tissue damage, PAS-stained kidney sections were evaluated as previously described.⁷ The frequency of glomerular crescent formation on day 7 of NTN was significantly increased in Treg-depleted DEREg mice compared with nephritic DEREg mice without DTx injection (Figure 3b (%): wt + DTx: 1.7 ± 1.7, DEREg + DTx: 1.7 ± 1.0, wt NTN: 16.1 ± 3.2, DEREg NTN: 16.1 ± 3.2, wt NTN + DTx: 15.0 ± 3.3, DEREg NTN + DTx: 29.5 ± 3.6; **P* < 0.05). As a measure of tubulointerstitial injury, an increase of the interstitial area estimated by point counting was observed in all nephritic groups. It did, however, not reveal differences with respect to Treg depletion (data not shown). The same results were obtained for urinary albumin/creatinine ratio (DEREG NTN vs DEREg NTN + DTx: 136.4 ± 15.8 vs 133.7 ± 19.6). Furthermore, blood urea nitrogen was elevated in all nephritic groups compared with controls, with a tendency to a further increase in Treg-depleted mice (Figure 3c (mg/dl): DEREg NTN vs DEREg NTN + DTx: 47 ± 5.6 vs 57.9 ± 10.4; *P* = 0.33).

Increased renal T-cell and macrophage/monocyte/dendritic cell (DC) recruitment in Treg-depleted nephritic mice

To investigate the role of Tregs regarding recruitment of T effector cells into the inflamed kidney, we performed immunohistological stainings of kidney sections for the pan T-cell marker CD3 in Treg-depleted and nondepleted

nephritic DEREg and wt mice (Figure 4a). At 7 days after induction of NTN, nephritic mice showed an increased renal infiltration of CD3⁺ T cells, predominantly in the interstitial and periglomerular area. Moreover, the frequency of intraglomerular T cells in nephritic mice was increased compared with nonnephritic controls. Interestingly, T-cell infiltrates in Treg-depleted nephritic mice were significantly increased in contrast to nondepleted nephritic mice (Figure 4b (cells per high power field): wt + DTx: 13.0 ± 2.0, DEREg + DTx: 22.7 ± 1.3, wt NTN: 22.5 ± 6.0, DEREg NTN: 32.5 ± 5.4, wt NTN + DTx: 23.5 ± 3.6, DEREg NTN + DTx: 62.3 ± 10.4; **P* < 0.05). This suggests an important role of Tregs regarding inhibition of T effector cell recruitment into the inflamed kidney. We also analyzed the number of F4/80⁺ macrophages/monocytes and DCs in kidney sections of healthy control and nephritic mice with and without Treg depletion (Figure 4c). Similarly, we detected a general increase of macrophage/monocyte/DC infiltrates in nephritic mice in contrast to the healthy control group. Treg depletion resulted in further enhancement of macrophage/monocyte/DC recruitment into the kidney (Figure 4d (cells per high power field): wt + DTx: 9.0 ± 1.5, DEREg + DTx: 10.7 ± 1.7, wt NTN: 14.0 ± 2.6, DEREg NTN: 26.3 ± 3.6, wt NTN + DTx: 17.3 ± 1.9, DEREg NTN + DTx: 42.3 ± 5.3; **P* < 0.05).

Upregulation of renal IFN γ response upon Treg depletion and NTN induction

To assess the renal T helper cell response on day 7 upon NTN induction, we first isolated RNA from renal cortex of nephritic DEREg and wt mice as well as the corresponding

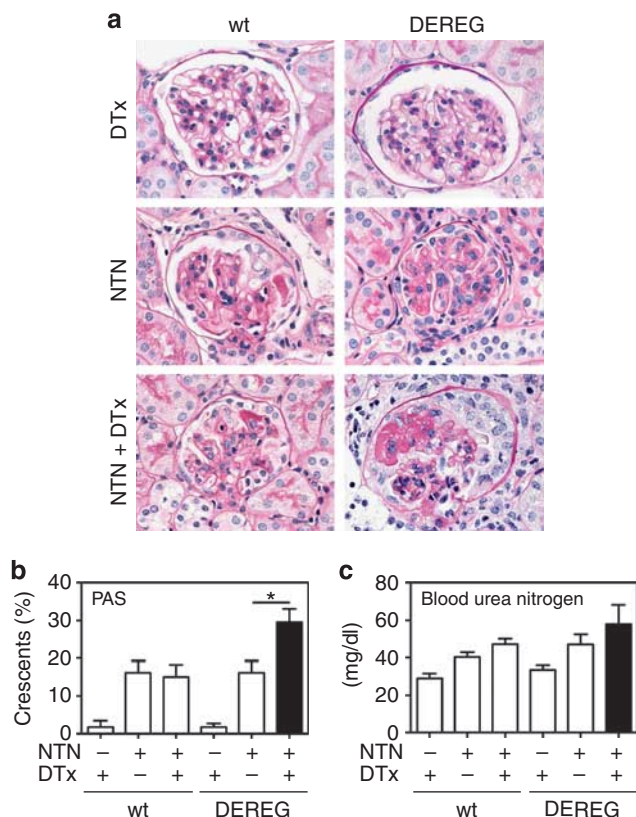


Figure 3 | Aggravated nephrotic nephritis (NTN) in regulatory T cell (Treg)-depleted DERE (depletion of regulatory T cell) mice. (a) Representative photographs of periodic acid-Schiff (PAS)-stained kidney sections of control mice (wild-type (wt) + diphtheria toxin (DTx)) and nephritic wt or DERE mice without or with DTx treatment (original magnification $\times 400$) on day 7 after induction of NTN. (b) Quantification of glomerular crescents ($*P < 0.05$) was performed. (c) Blood urea nitrogen (BUN) was determined.

controls and measured the expression of Th1 and Th17 effector cytokines. $\text{IFN}\gamma$ mRNA expression was significantly upregulated in kidney tissue of Treg-depleted nephritic DERE mice in contrast to nondepleted nephritic mice (Figure 5a: $\text{IFN}\gamma$: wt + DTx: 0.8 ± 0.1 , DERE + DTx: 2.0 ± 0.8 , wt NTN: 1.0 ± 0.2 , DERE NTN: 1.1 ± 0.3 , wt NTN + DTx: 1.4 ± 0.2 , DERE NTN + DTx: 7.0 ± 1.6 ; $***P < 0.0001$). IL-17 was generally upregulated in nephritic mice. However, in contrast to $\text{IFN}\gamma$, renal expression of IL-17 was unchanged in Treg-depleted and nondepleted nephritic mice (Figure 5a). The increased $\text{IFN}\gamma$ expression induced by ablation of Tregs correlated with elevated CXCL10 (chemokine (C-X-C motif) ligand 10) expression (data not shown), one of the ligands for CXCR3 (chemokine (C-X-C motif) receptor 3) receptors that mainly mediate the migration of Th1 cells into the kidney.⁹

To verify the Treg-mediated suppression of $\text{IFN}\gamma$ responses, we performed intracellular cytokine staining in renal CD4^+ T cells from control and nephritic mice after *in vitro* stimulation with phorbol 12-myristate 13-acetate/ionomycin. Corresponding to the elevated $\text{IFN}\gamma$ mRNA expression in kidney tissue, the $\text{IFN}\gamma$ protein expression was

strongly increased in renal T cells isolated from Treg-depleted nephritic mice compared with nondepleted nephritic mice (Figure 5b: 43.6 vs 17.4%). Again, protein expression of IL-17 remained nearly unchanged between 2 and 3% in all groups of nephritic mice (Figure 5b). Quantification of three independent FACS analyses displayed a significant increase of $\text{IFN}\gamma$ - but not IL-17-producing CD4^+ T cells in Treg-depleted nephritic DERE mice in contrast to nondepleted nephritic DERE or wt mice (Figure 5c: $\text{IFN}\gamma^+ \text{CD4}^+$ T cells (%): wt NTN: 17 ± 1.4 , DERE NTN: 15.8 ± 1.4 , wt NTN + DTx: 18.5 ± 0.7 , DERE NTN + DTx: 35.5 ± 4.3 ; $***P < 0.0001$).

To evaluate whether the absence of Tregs during the induction phase of nephritis is sufficient for the aggravated phenotype in Treg-depleted mice, we directly compared a single injection of DTx on day -1 with the continuous Treg depletion (days -1 and 3; Supplementary Figure S1 online). Interestingly, early depletion of Tregs resulted in a sustained reduction of splenic Tregs from 16.7 to 5.9% up to day 7, whereas a double DTx injection was necessary for complete depletion of splenic Tregs (Supplementary Figure S1A online: $\text{CD4}^+ \text{FoxP3}^+ \text{GFP}^+$ T cells (%): 16.7 ± 0.75 vs 2.9 ± 0.5 ; $***P < 0.0001$). Accordingly, glomerular damage was only slightly elevated in mice with a single early depletion but was considerably aggravated in continuously depleted mice (Supplementary Figure S1B online).

Augmented systemic immune response in Treg-depleted nephritic mice

To investigate the role of endogenous $\text{CD4}^+ \text{CD25}^+ \text{FoxP3}^+$ Tregs regarding the systemic immune response in the mouse model of NTN, splenocytes were isolated from wt and DERE mice 7 days after induction of NTN and stimulated *in vitro* with sheep immunoglobulin G (IgG), the nephritogenic antigen. Consistent with the results obtained in the inflamed target organ, namely in the kidney, ELISA analysis of splenocyte supernatants indicated that Treg depletion in nephritic mice resulted in an increased systemic $\text{IFN}\gamma$ secretion compared with NTN mice without Treg depletion (Figure 6a, $\text{IFN}\gamma$ (pg/ml): wt + DTx: 1.0 ± 0.02 , DERE + DTx: 1.0 ± 0.02 , wt NTN: 421.0 ± 54.5 , DERE NTN: 608.7 ± 37.2 , wt NTN + DTx: 307.0 ± 37.9 , DERE NTN + DTx: 3457.0 ± 175.8 ; $***P < 0.0001$). IL-17 secretion by splenocytes from mice with NTN was not elevated in Treg-depleted nephritic mice (Figure 6a, IL-17 (pg/ml): wt + DTx: 1.0 ± 0.02 , DERE + DTx: 1.0 ± 0.02 , wt NTN: 39.3 ± 3.7 , DERE NTN: 45.7 ± 3.8 , wt NTN + DTx: 60.8 ± 3.0 , DERE NTN + DTx: 25.0 ± 2.1 ; $***P < 0.0001$).

Moreover, systemic humoral immune responses to sheep IgG are important for initiation of the autologous phase of NTN. Hence, we determined the IgG antibody response directed against the nephritogenic antigen in serum samples by ELISA for sheep IgG-specific mouse IgG subclasses. Depletion of Tregs resulted in significantly increased levels of total IgG and the isotypes of IgG1, IgG2a/c,¹⁰ and IgG2b (Figure 6b).

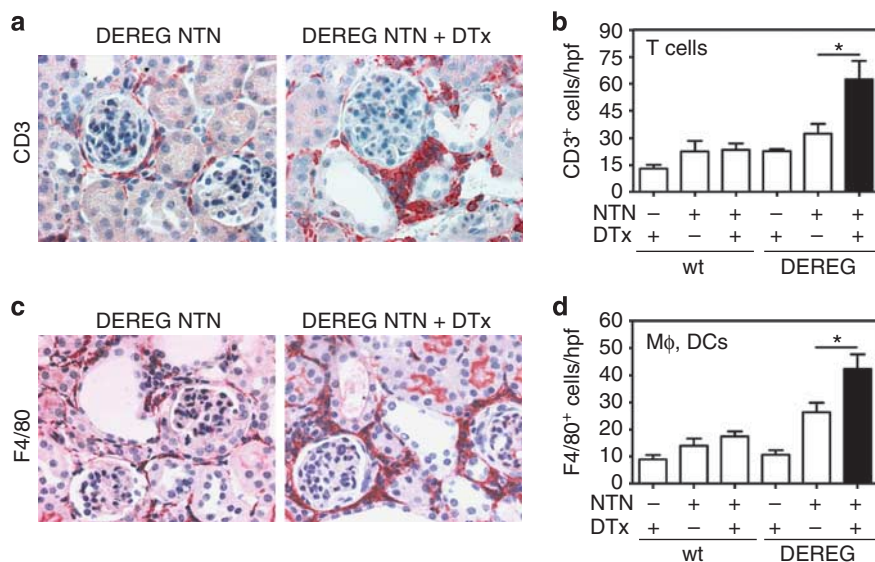


Figure 4 | Increased renal T-cell and macrophage (M ϕ)/monocyte/dendritic cell (DC) recruitment in regulatory T cell (Treg)-depleted nephritic mice. Representative photographs of kidney sections from nephritic DEREK (depletion of regulatory T cell) mice with or without diphtheria toxin (DTx) treatment were immunohistochemically stained for the (a) T-cell marker CD3 and the (c) monocyte/macrophage/DC marker F4/80, 7 days after nephrotoxic nephritis (NTN) induction (original magnification $\times 400$). (b) Quantification of tubulointerstitial and glomerular CD3⁺ T cells in nephritic mice without or with DTx treatment and (d) tubulointerstitial F4/80⁺ cells per high power field (hpf) was assessed (* $P < 0.05$).

Furthermore, we performed immunohistochemical staining of kidney sections for sheep IgG (Supplementary Figure S2A online) and mouse IgG (Supplementary Figure S2B online) to evaluate the amount of antibody deposition in injured glomeruli. Semiquantitative assessment of mouse IgG-stained sections revealed that discrete granular mouse IgG immune deposit within the glomeruli were increased in nephritic mice but were not further elevated in nephritic Treg-depleted mice compared with nephritic mice without depletion (Supplementary Figure S2B online).

Reduced disease severity in Treg-depleted nephritic mice upon anti-IFN γ treatment

To investigate the role of endogenous Tregs regarding Treg-mediated suppression of Th1 immune responses, we inhibited the Th1-specific IFN γ response by injection of an anti-IFN γ antibody together with the first DTx dose at 1 day before NTN induction in DEREK mice. Indeed, aggravation of glomerular damage as measured by a significant increase of crescent formation in Treg-depleted nephritic controls was significantly inhibited in Treg-depleted, anti-IFN γ -treated nephritic mice (Figure 7a (%): wt: 1.1 ± 0.6 , DEREK NTN: 27 ± 1.3 , DEREK NTN + DTx: 39.5 ± 4.2 , DEREK NTN + α IFN γ : 19.5 ± 2.3 , DEREK NTN + DTx + α IFN γ : 26.3 ± 1.4 ; ** $P < 0.01$). However, blood urea nitrogen was elevated in all nephritic groups compared with controls, but did not show a significant reduction in anti-IFN γ -treated mice (Figure 7b). FACS analysis of renal T cells indicated that anti-IFN γ treatment reversed the enhanced frequency of IFN γ -producing CD4⁺ Th1 cells in Treg-depleted nephritic mice on day 7 upon NTN induction (Figure 7c; 28.6 vs 48%).

Concordantly, renal expression of T-bet, a Th1-specific transcription factor, as well as of the IFN γ -inducible chemokine CXCL10, which recruits CXCR3⁺ Th1 cells, were significantly inhibited upon anti-IFN γ treatment (Figure 7d: CXCL10: wt: 1 ± 0.08 , DEREK + NTN: 7.5 ± 0.8 , DEREK NTN + DTx: 19.5 ± 2.8 , DEREK NTN + α IFN γ : 8 ± 0.9 , DEREK NTN + DTx + α IFN γ : 11.3 ± 0.4 ; T-bet: wt: 1 ± 0.24 , DEREK + NTN: 2.7 ± 0.4 , DEREK NTN + DTx: 7.9 ± 0.9 , DEREK NTN + α IFN γ : 2.1 ± 0.35 , DEREK NTN + DTx + α IFN γ : 4.1 ± 1.3 ; *** $P < 0.0001$; * $P < 0.05$). Moreover, measurement of the systemic humoral anti-sheep immune response in mouse serum indicated that the IFN γ -related IgG2a/c response was significantly inhibited in anti-IFN γ -treated Treg-depleted nephritic DEREK mice compared with the nontreated ones (Figure 7e (optical density at 450 nm): wt: 0.17 ± 0.01 , DEREK + NTN: 0.39 ± 0.03 , DEREK NTN + DTx: 1.36 ± 0.18 , DEREK NTN + α IFN γ : 0.37 ± 0.03 , DEREK NTN + DTx + α IFN γ : 0.87 ± 0.16 ; *** $P < 0.0001$; * $P < 0.05$). In contrast, concentrations of the 'non-Th1-related' antibodies IgG1 and IgG2b remained unaffected (Figure 7e).

DISCUSSION

Tregs develop in the thymus in order to regulate autoimmunity in the periphery. Upon inflammation, they become activated and increase in number, either by proliferation of thymus-derived naturally occurring natural Tregs and/or by peripheral conversion of CD4⁺CD25⁻ responder T cells to CD4⁺CD25⁺FoxP3⁺ Tregs.¹¹ Here we describe that upon induction of experimental crescentic glomerulonephritis, frequency as well as absolute number of

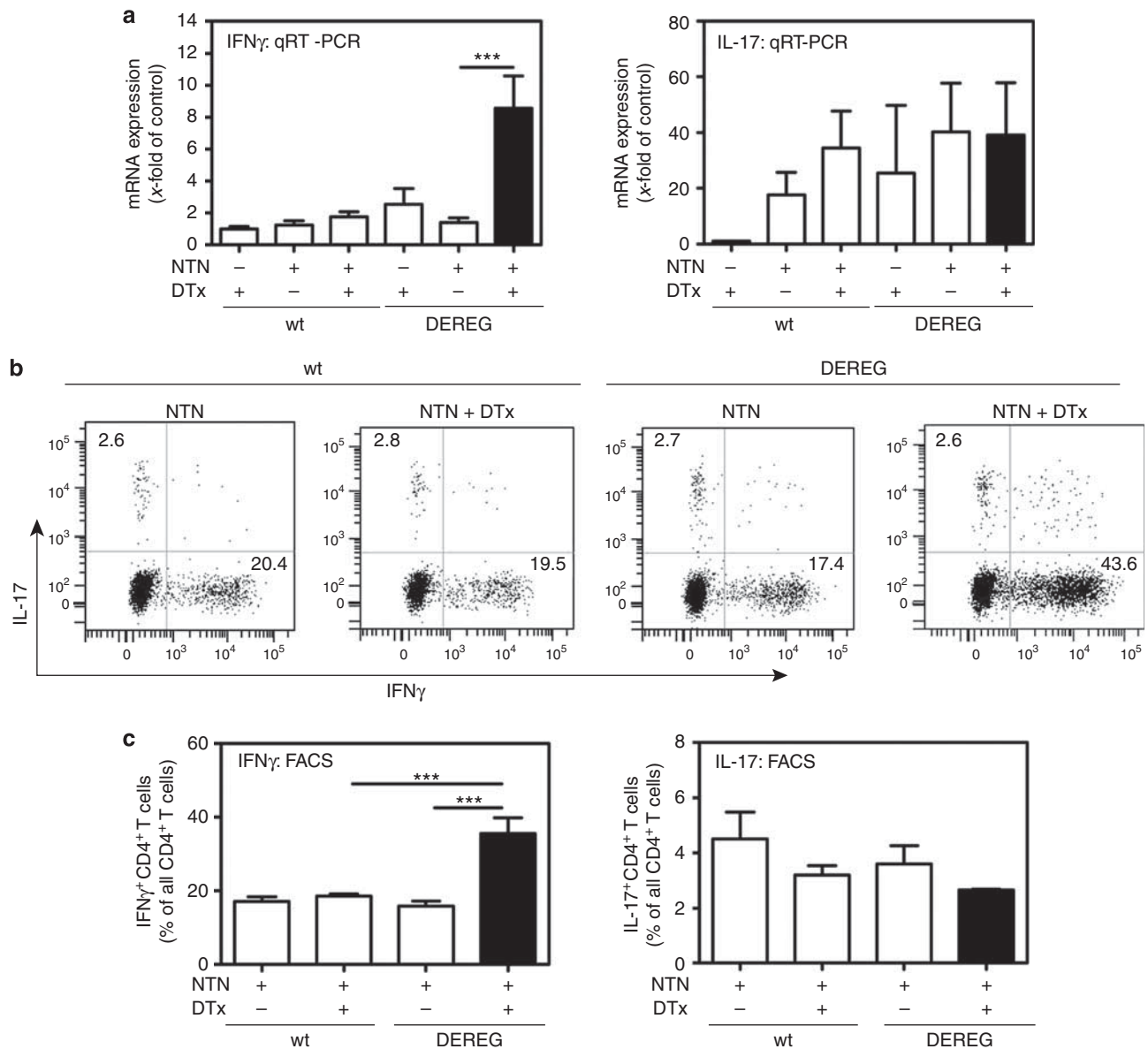


Figure 5 | Upregulation of renal Th1 response upon regulatory T cell (Treg) depletion in nephritic mice. (a) Total RNA was extracted from kidneys of wild-type (wt) and DEREg (depletion of regulatory T cell) mice with/without nephrotoxic nephritis (NTN) \pm diphtheria toxin (DTx) treatment. Subsequently, quantitative real-time reverse transcription (RT)-PCR was performed for interferon- γ (IFN γ) and interleukin-17 (IL-17) expression (** $P < 0.0001$). The expression levels are indicated as x-fold of nonnephritic wt controls. (b) Renal single-cell suspensions from wt and DEREg mice without or with NTN \pm DTx treatment were stimulated *in vitro* with phorbol 12-myristate 13-acetate (PMA)/ionomycin. Intracellular cytokine production of IFN γ and IL-17 was analyzed by flow cytometry. Representative dot plots are depicted. Cells are gated on CD4⁺ T cells and numbers represent events in quadrants in percentage of all gated events. (c) The frequencies of IFN γ - and IL-17-producing renal CD4⁺ T cells were quantified (** $P < 0.0001$). All analyses were performed at day 7 after induction of NTN.

FoxP3⁺ Tregs increased substantially within renal tissue and more weakly within spleen.

Depletion of Tregs by repetitive injections of DTx on day -1 and day 3 upon NTN induction resulted in exacerbation of glomerular crescent formation and mouse anti-sheep IgG antibody production as well as in enhanced systemic and renal IFN γ production. However, depletion of Tregs only during the heterologous phase of NTN nephritis, that is, when antigen-specific priming is likely to occur in the lymphoid tissue, failed to increase crescent formation

(Supplementary Figure S1B online). We observed similar results by starting Treg depletion at the beginning of the autologous phase (data not shown). Hence, it seems likely that both Tregs that home in the lymphoid tissue and Tregs that migrate into the inflamed kidney control glomerulonephritis. The enhanced IFN γ response observed in the aggravated phenotype correlated with an increase of macrophage/monocyte/DC and T-cell infiltration into periglomerular and interstitial areas of the renal cortex. As Tregs were depleted in this experimental setting, the infiltrating CD3⁺

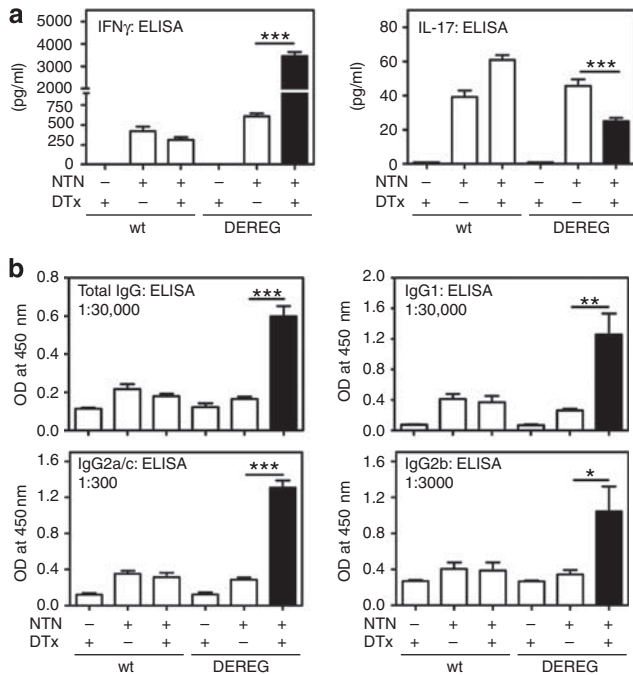


Figure 6 | Enhanced systemic Th1 immune response in regulatory T cell (Treg)-depleted mice. (a) Cytokine secretion of interferon- γ (IFN γ) and interleukin-17 (IL-17) in supernatants of cultured splenocytes after treatment with sheep immunoglobulin G (IgG) was measured by enzyme-linked immunosorbent assay (ELISA; $***P < 0.0001$). (b) Circulating titers of mouse anti-sheep total IgG and isotypes of IgG1, IgG2a/c, and IgG2b at day 7 after induction of nephritis were measured by ELISA in wild-type (wt) and DEREg (depletion of regulatory T cell) mice without and with nephrotoxic nephritis (NTN) and diphtheria toxin (DTx) treatment. Serum was diluted as indicated ($***P < 0.0001$, $**P < 0.01$, $*P < 0.05$).

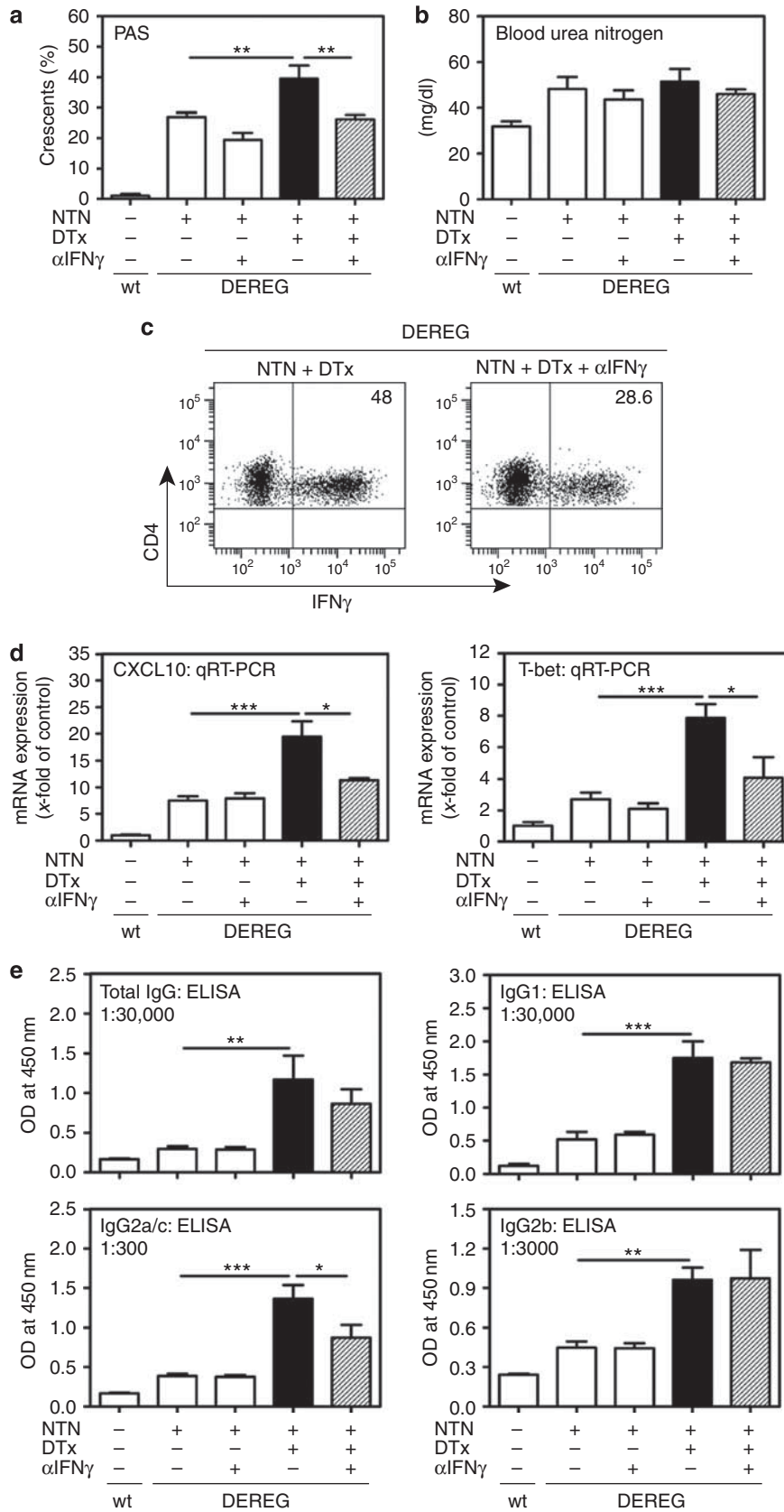
T cells most likely consisted of activated effector T lymphocytes of which the CD4⁺ cells indeed produced substantially increased amounts of IFN γ whereas IL-17 production remained unaffected, although glomerular injury in our model is mediated by a Th1 as well as by a Th17 response.^{9,12} However, it seems noteworthy that the systemic release of IL-17 detected in supernatants of splenocyte cultures was significantly suppressed in the Treg-depleted group. In view of the fact that Tregs also suppress maturation and function of DCs,¹³ this can be explained by the observation that IL-12, the major inducer of IFN γ , decreases IL-17 production.¹⁴ Intriguingly, a recent publication also showed that Tregs from patients with active pulmonary tuberculosis suppressed

IFN γ production, whereas the Th17 response remained unaffected.¹⁵ Taken together, the most dramatic effect of Treg depletion observed in our study was aggravation of the IFN γ T-cell response. These results suggest that the observed increase of frequency and absolute number of immunosuppressive Tregs during acute NTN regulates the systemic and local inflammatory Th1 response.

Noteworthy, in our experiments DEREg mice followed the equivalent characteristic features of NTN compared with C57BL/6 wt mice. Furthermore, DTx neither caused renal toxicity in wt or DEREg mice nor nonresponsiveness toward itself,¹⁶ as PAS staining failed to show histopathological alterations and DTx efficiently depleted Tregs as measured 4 days after the second injection, respectively.

Up to now, three reports dealt with suppressive effects of Tregs in the planted antigen model of nephrotoxic serum nephritis. Adoptive transfer of CD4⁺CD25⁺ Tregs protected mice from nephritis induced by anti-glomerular basement membrane rabbit serum. Measuring tissue levels of FoxP3 mRNA, the authors concluded that most of the adoptively transferred Tregs migrated to secondary lymphoid organs but not into the inflamed kidney.⁵ However, using FACS analysis and quantitative immunohistochemistry, we could recently demonstrate that adoptively transferred Tregs migrate into the kidney as long as they express the chemokine receptor CCR6,⁷ and CCR6 expression by adoptively transferred Tregs was absolutely necessary for their protective effect. Hence, together with the finding that Treg depletion only in the heterologous phase failed to induce the aggravated phenotype, it seems that CD4⁺FoxP3⁺ Tregs have to accumulate within the inflamed kidney, at least in dependence of CCR6, in order to sufficiently suppress glomerulonephritis. Results of the third report suggest that lymphoid homing of Tregs is a prerequisite for downregulation of anti-glomerular basement membrane nephritis.⁶ Our results presented here show that in contrast to spleen, CD4⁺FoxP3⁺ Tregs substantially increased within kidney during NTN nephritis in comparison with normal frequency of ~5% in healthy mice, and depletion of these cells significantly correlated with aggravation of glomerulonephritis. The necessity of inflammatory tissue-homing Tregs for suppression of inflammatory tissue damage has also been observed in models of intestinal inflammation¹⁷ and inflammatory liver injury.¹⁸ Taken together, these data indicate that the anti-inflammatory functions of Tregs in experimental glomerulonephritis take

Figure 7 | Attenuation of renal and systemic Th1 immune response upon neutralization of interferon- γ (IFN γ). (a) Quantification of glomerular crescent formation in depleted or nondepleted nephritic DEREg (depletion of regulatory T cell) mice without or with administration of anti-IFN γ antibody ($**P < 0.01$). (b) Blood urea nitrogen (BUN) levels were determined in nonnephritic wild-type (wt) littermates and nephritic DEREg mice. (c) Renal single-cell suspensions from wt mice and nephritic DEREg mice \pm diphtheria toxin (DTx) \pm α IFN γ treatment were stimulated *in vitro* with phorbol 12-myristate 13-acetate (PMA)/ionomycin. Intracellular cytokine production of IFN γ was analyzed by flow cytometry. Representative dot plots are depicted. Cells are gated on CD4⁺ T cells and numbers represent events in quadrants in percentage of all gated events. (d) Quantitative real-time PCR analysis of renal mRNA expression of CXCL10 (chemokine (C-X-C motif) ligand 10) and T-bet was performed in nephritic DEREg mice (\pm DTx; \pm α IFN γ) and wt littermates ($***P < 0.0001$, $*P < 0.05$). The mRNA levels are expressed as \times -fold of nonnephritic wt controls. (e) Circulating titers of mouse anti-sheep total immunoglobulin G (IgG) and isotypes of IgG1, IgG2a/c, and IgG2b were measured by enzyme-linked immunosorbent assay (ELISA) in wt mice and DEREg nephrotoxic nephritis (NTN) mice with or without treatment of DTx and/or α IFN γ . Serum was diluted as indicated ($***P < 0.0001$, $**P < 0.01$, $*P < 0.05$). All analyses were performed 7 days after NTN induction.



place in the inflamed kidney itself and in the secondary lymphatic organs such as the renal lymph nodes.

In addition to anti-glomerular basement membrane nephritis,⁵ adoptively transferred Tregs have been shown to prevent murine lupus nephritis.¹⁹ However, Treg cells did not only suppress 'immune-mediated' glomerulonephritis, but also 'non-immune' toxin-mediated kidney injury in the adriamycin nephropathy model. In this model, polyclonal CD4⁺CD25⁺ Tregs²⁰ and, moreover, FoxP3-transduced T cells,²¹ $\gamma\delta$ T cells, or alternatively activated macrophages³ were approved as efficient immunotherapy against renal injury. As anti-inflammatory cytokines represent one critical mechanism of regulatory immune cells including Tregs, these findings support the concept that Tregs not only suppress adaptive T-cell responses but also pathology driven by innate immune mechanisms.²² Hence, the immunosuppressive mechanisms by which Tregs suppress glomerulonephritis resulting from different etiologies remain to be elucidated.

In patients with autoimmune kidney disease such as lupus nephritis, decreased proportions of Tregs have been reported to inversely correlate with clinical disease activity.^{23,24} However, several studies also reported unaltered or even increased numbers of Tregs in lupus patients and positive correlation with disease activity. Similar results were obtained when the functional immunosuppressive activity of Tregs from lupus patients was assessed. These divergent results can be explained by the fact that in contrast to the murine system, in humans FoxP3 is rather an activity marker for T cells than a specific marker for Tregs. Although one can imagine that Treg numbers and function are impaired during chronic disease, our results shown here demonstrate that during acute glomerulonephritis, Treg numbers initially increased and their immunosuppressive function was at least as effective as the function of Tregs from healthy controls.

In conclusion, depletion of Tregs exacerbated acute kidney inflammation and glomerular injury, an effect that was most likely mediated by IFN γ , as IFN γ production by intrarenal and splenic CD4⁺ T cells substantially increased and anti-IFN γ antibodies significantly suppressed this effect. Hence, this study might be useful to evaluate novel therapeutic approaches by modulating Tregs in order to suppress Th1 immune responses in kidney diseases.

MATERIALS AND METHODS

Animals

Male DREG mice expressing the DTx receptor and GFP under control of the FoxP3 promoter⁸ and sex/age-matched (8–10 weeks old) C57BL/6 wt controls were obtained from animal facilities of the Universitätsklinikum Hamburg-Eppendorf. Animals received humane care according to guidelines of the National Institute of Health in Germany. Experiments were approved by the institutional review board 'Behörde für Soziales, Familie, Gesundheit und Verbraucherschutz' (Hamburg).

Animal treatment and functional studies

Nephrotoxic serum nephritis was induced in C57BL/6 wt and DREG mice by intraperitoneal injection of 500 μ l of nephrotoxic sheep serum

per mouse. Controls were injected intraperitoneally with an equal amount of nonspecific sheep IgG. To deplete Tregs in DREG mice, DTx (1 μ g per mouse) was injected intraperitoneally either twice 24 h before NTN induction and 3 days upon NTN induction or only once 24 h before NTN induction. Rat anti-mouse IFN γ monoclonal antibody (XGM1.2; see Mumberg *et al.*²⁵); 500 μ g/mouse) or rat IgG was injected intraperitoneally 24 h before NTN induction. Blood samples for measurement of blood urea nitrogen and assessment of systemic antibody response were obtained at the time of killing. For urine sample collection, mice were housed in metabolic cages for 6 h. Urinary creatinine and blood urea nitrogen were measured by standard laboratory methods. Albuminuria was determined by standard ELISA analysis (mice-albumin kit; Bethyl Laboratories, Montgomery, TX).

Real-time quantitative reverse transcription-PCR analysis

Total RNA was reversely transcribed followed by quantitative reverse transcription-PCR using BIORAD CFX96 real-time system and ABsoluteQPCR SYBR mix (Fisher Scientific GmbH, Schwerte, Germany). Primer pairs were used as described previously.^{7–9} Relative mRNA levels were calculated after normalization to 18S rRNA using the CFX96 Manager software (Bio-Rad Laboratories, Munich, Germany).

Morphological examinations

Light microscopy and immunohistochemistry were performed by routine procedures. Crescent formation was assessed in 30 glomeruli per mouse in a blinded fashion in PAS-stained paraffin sections. Tubulointerstitial injury was estimated by point counting in four independent areas of renal cortex per mouse in 200-fold magnification.⁷ Paraffin-embedded sections (2 μ m) were either stained with an antibody directed against the pan-T-cell marker CD3 (A0452; Dako, Hamburg, Germany), the Treg transcription factor FoxP3 (FJK-16s; eBioscience, San Diego, CA), the macrophage/monocyte/DC marker F4/80 (BM8; BMA, Augst, Switzerland), and sheep or mouse IgG (Jackson ImmunoResearch Europe, Newmarket, UK). Tissue sections were developed with the Vectastain ABC-AP kit (Vector Laboratories, Burlingame, CA). For FoxP3 staining, tissue sections were incubated with a polyclonal rabbit anti-rat secondary antibody (Dako) and developed with the ZytocemPlus (AP) Polymer Kit (Zytomed Systems, Berlin, Germany). CD3⁺ cells in 20 glomerular cross-sections and F4/80⁺ and CD3⁺ cells in 20 tubulointerstitial high power fields (\times 400) per kidney were counted in a blinded fashion. For quantification of FoxP3⁺ Tregs, at least seven low power fields (\times 100) were counted. Glomerular mouse IgG deposition was scored from 0 to 3 in 20 glomeruli per mouse as previously described.⁷

Antigen-specific humoral immune response

Mouse anti-sheep IgG antibody titers were measured by ELISA using sera collected 7 days after induction of nephritis.^{7,9,26} In brief, ELISA microtiter plates were coated with 100 μ l sheep IgG (100 μ g/ml; Sigma, St Louis, MO) in carbonate-bicarbonate buffer overnight at 4 °C. After blocking with 1% bovine serum albumin in Tris-buffered saline (Sigma), plates were incubated with serial dilutions of mouse serum for 1 h at room temperature. Bound mouse IgG was detected using peroxidase-conjugated goat anti-mouse IgG (Biozol, Eching, Germany), TMB peroxidase substrate, and absorbance readings (450 nm). Lack of crossreactivity of the secondary antibody with sheep IgG was demonstrated by omitting the primary antibody. The bound mouse immunoglobulin isotypes were detected using peroxidase-conjugated rabbit anti-mouse IgG, IgG1, IgG2a/c, and IgG2b antibodies (Zymed-Invitrogen, Karlsruhe, Germany).

Leukocyte isolation from various tissues

Previously described methods for leukocyte isolation from murine kidneys were used.^{7,26} In brief, kidneys were finely minced and digested for 45 min at 37 °C with 0.4 mg/ml collagenase D (Roche, Mannheim, Germany) and 0.01 mg/ml DNase I in Dulbecco's modified Eagle's medium (Roche) supplemented with 10% heat-inactivated fetal calf serum (Invitrogen, Darmstadt, Germany). Cell suspensions were sequentially filtered through 70 and 40 µm nylon meshes and washed with Hank's balanced salt solution without Ca²⁺ and Mg²⁺ (Invitrogen). Single-cell suspensions were separated using Percoll density gradient (70 and 40%) centrifugation. Single-cell suspensions of spleens were prepared according to standard laboratory procedures. In brief, tissue was passed through 70 and 40 µm nylon meshes before erythrocyte lysis. Subsequently, cells were washed several times with Hank's balanced salt solution and resuspended in RPMI-1640 with 10% fetal calf serum. Viability of the cells was assessed by Trypan blue staining before flow cytometry.

Flow cytometry

Leukocytes were stained using a standard protocol. For T-cell differentiation, isolated cells were stained with anti-CD3 (APC; eBioscience), anti-CD4 (APC-AlexaFluor750), and anti-CD45 (PerCP; both from BD Biosciences, Franklin Lakes, NJ) upon a blocking step. Staining of intracellular IFN γ and IL-17 was performed as described previously.¹² In brief, splenocytes or renal leukocytes were stimulated with phorbol 12-myristate 13-acetate (50 ng/ml; Sigma) and ionomycin (1 µg/ml; Calbiochem-Merck, Darmstadt, Germany) for 5 h. After 30 min of incubation, Brefeldin A (10 µg/ml; Sigma) was added. After several washing steps and staining of cell surface markers, cells were permeabilized using Cytofix/Cytoperm (BD Biosciences). Subsequently, intracellular staining was performed using rat anti-mouse IFN γ and IL-17 antibodies (V450 or PE; BD Biosciences) and an anti-FoxP3 antibody (eBioscience). Data were recorded using BD LSRII Flow Cytometry system and BD FACSDiva software.

Isolation and culture of splenic CD4⁺CD25⁺ Tregs and responder T cells

Spleens were excised from C57BL/6 wt 8 days after induction of NTN, and from healthy controls and passed through 100 µm nylon meshes. Sorting procedures were carried out by MACS according to the manufacturers' instructions (MACS CD4⁺ T-Cell-Isolation Kit; Miltenyi Biotec, Bergisch-Gladbach, Germany). Briefly, CD4⁺ T cells were enriched using a biotinylated antibody cocktail depleting all other blood cell types and anti-biotin microbeads. CD4⁺CD25⁺ T cells were isolated by positive selection using phycoerythrin-labeled anti-CD25 monoclonal antibody and anti-phycoerythrin microbeads. Purity and intracellular FoxP3 expression was controlled by flow cytometry. Next, 1 × 10⁵ wt responder T cells (CD4⁺CD25⁻) isolated from healthy mice were cultured alone or with 1 × 10⁵ CD4⁺CD25⁺ Tregs from nephritic wt or healthy controls for 72 h in 96-well plates precoated with anti-CD3 monoclonal antibody (5 µg/ml; clone 145-2C11; BD Biosciences). Cytokine concentrations were measured in supernatants by ELISA.

Statistical analysis

Results are expressed as mean ± s.e.m. Differences between individual experimental groups were compared by Student's *t*-test. In case of multiple comparisons, one-way analysis of variance with post analysis by Tukey-Kramer test was used. Experiments that did not

yield enough independent data for statistical analysis due to the experimental setup were repeated at least three times.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Figure S1 Correlation of efficiency of Treg depletion and crescent formation upon single or double injection of DTx to DREG mice.

Figure S2. Semiquantitative scoring of glomerular mouse IgG deposition.

Supplementary material is linked to the online version of the paper at <http://www.nature.com/ki>

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