$CD4^+CD25^+$ Regulatory T Cells Inhibit Experimental Anti–Glomerular Basement Membrane Glomerulonephritis in Mice

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$CD4^+CD25^+$ regulatory T cells (Treg) are of critical importance for the maintenance of tolerance. The kidney is frequently involved in autoimmune diseases, such as lupus erythematosus or glomerulonephritis (GN). Therefore, the therapeutic efficacy of Treg in a T cell–dependent murine model of experimental anti–glomerular basement membrane (anti-GBM) GN was tested. Transfer of 1×10^6 $CD4^+CD25^+$ T cells (day −1) into mice that were previously immunized with rabbit IgG (day −3) and subsequently received an injection of anti-GBM rabbit serum (day 0) significantly attenuated the development of proteinuria when compared with animals that received an injection of 1×10^6 $CD4^+CD25^-$ T cells (control group). Treg injection induced a dramatic decrease of glomerular damage as well as a marked decrease of $CD4^+$ T cell, $CD8^+$ T cell, and macrophage infiltration. Of note, deposition of immune complexes was not prevented by Treg, showing that Treg rather inhibited cell-mediated organ damage than priming of the humoral immune response. Accordingly, a significant reduction of IFN-γ, TNF-α, and TGF-β1 mRNA in kidneys from animals that received Treg injection was observed. Tracking of enhanced green fluorescence protein–transgenic Treg revealed a predominant migration to secondary lymphoid organs with a significant increase of regulatory T cells ($CD4^+CD25^+CD69^-CD45RB^{low}$) in the lymph nodes. In contrast, enhanced green fluorescence protein– and FoxP3-positive cells by reverse transcription–PCR and $CD4^+CD25^+CD69^-CD45RB^{low}$ T cells by flow cytometry in the kidney of nephritic animals were not detected. This report provides first evidence that Treg are potent suppressors of anti-GBM GN. Treg therefore might be of therapeutic value for the treatment of severe GN in humans.

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Human glomerulonephritis (GN) is characterized by a wide spectrum of clinical presentations, histologic patterns, and functional outcomes. Studies in human GN and in experimental models now have confirmed a central role for T cells and T cell–directed effector mechanisms in the development and progression of glomerular injury (1,2). Priming of T cells requires antigen presentation via the MHC II complex, which is expressed on professional antigen presenting cells (APC) such as dendritic cells and macrophages. T cell priming is known to be induced primarily in secondary lymphoid organs and is a prerequisite for the development of crescentic anti–glomerular basement membrane (anti-GBM) GN in mice (3). In line with this suggestion, transfer of antigen-specific nephritogenic T cells is sufficient for the development of GN in healthy animals (1,4,5).

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Recently, $CD4^+CD25^+$ regulatory T cells (Treg) were described to play a pivotal role in the maintenance of tolerance in rodents and humans (6). Several experimental models of organ-specific autoimmune diseases provided convincing evidence that thymus-derived Treg maintain self-tolerance (7,8), as they protect from the development of autoimmune diseases (9,10). According to these findings from animal studies, patients who have autoimmune diseases display a numerical and functional Treg deficit (11,12). These data clearly show that under conditions of an impaired immune tolerance to autoantigens, a decrease of Treg number and/or function might promote the development of autoimmune diseases. In line with this suggestion, Powrie and colleagues (13,14) demonstrated that transfer of Treg protected mice from the development of inflammatory bowel disease and even reversed established gastrointestinal inflammation. The mechanism by which Treg regulate the immune response remains controversial so far (15). Importantly, Treg have to be distinguished from type 1 regulatory T (Tr1)-like cells, which protect from autoimmune diseases primarily by the production of immunosuppressive cytokines such as TGF-β, IL-10, and in some experimental models IL-4 (16–18). In contrast to Tr1-like cells, immunosuppression mediated by

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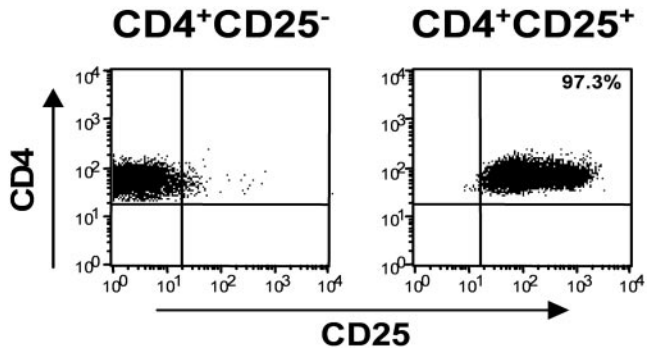

Figure 1. Purity of isolated $CD4^+CD25^-$ and $CD4^+CD25^+$ splenocytes. A representative flow cytometry analysis of the purity of both cell populations after the isolation procedure using magnetic beads as described in the Materials and Methods section is given.

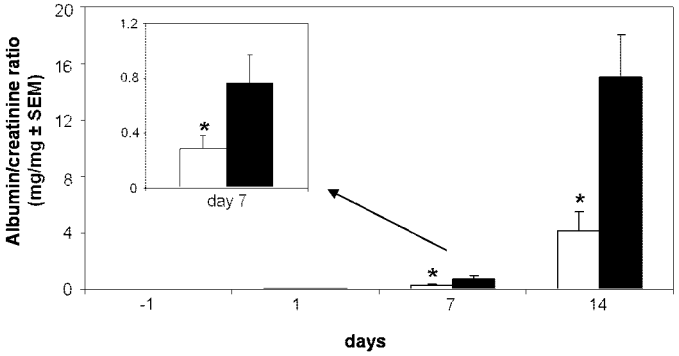

Figure 2. Transfer of regulatory T cells (Treg) significantly reduces proteinuria. Before and after induction of anti–glomerular basement membrane (anti-GBM) glomerulonephritis (GN) proteinuria was evaluated on days −1, 1, 7 ($n = 14$ per group), and 14 ($n = 7$ per group). Urine albumin excretion (in mg) was determined and expressed per milligram of urinary creatinine to standardize for the GFR. Mice that received control $CD4^+CD25^-$ T cells (■) had significant albuminuria, whereas mice after Treg transfer (□) showed only minimal albuminuria ($^*P < 0.05$).

Treg is known to be strictly dependent on cell-to-cell contact *in vitro* and *in vivo* (19,20).

The objective of the present study was to determine the influence of Treg transfer on the pathogenesis of GN in a complement-dependent Th1-predominant model of accelerated nephrotoxic nephritis (21). GN in mice that received $CD4^+CD25^-$ control cells was characterized by significant proteinuria, histologic indices of glomerular damage, and T cell and macrophage infiltration in the diseased kidney. In sharp contrast, animals that received an injection of Treg developed only minimal proteinuria, reflecting the functional consequence of a marked reduction of glomerular injury. In addition, the expression of IFN-γ, TNF-α, and TGF-β1 was significantly decreased in kidneys of Treg-treated animals. Notably, autologous antibody production was not influenced by Treg transfer. Treg were found primarily in secondary lymphoid organs but not in the kidney. Our current report demonstrates for the first time the potent immunosuppressive effect of Treg in anti-GBM GN in mice. These data provide evidence that Treg might be of therapeutic value for the treatment of severe autoimmune GN in humans.

Materials and Methods

Isolation of $CD4^+CD25^+$ Regulatory T Cells

$CD4^+CD25^+$ Treg and $CD4^+CD25^-$ control cells were isolated from minced spleens obtained from female C57Bl/6J mice (or when indicated from enhanced green fluorescence protein [EGFP]-transgenic CD57/Bl6 mice or from nephritic animals) using magnetic bead separation ($CD4^+CD25^+$ regulatory T cell kit; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Briefly, non-$CD4^+$ T cells were depleted using a biotinylated antibody cocktail and anti-biotin microbeads. $CD4^+CD25^+$ T cells were subsequently separated from $CD4^+CD25^-$ T cells by positive selection using PE-labeled anti-CD25 mAb and anti-PE microbeads. The purity of both populations was controlled by flow cytometric analysis and routinely reached >90% (Figure 1).

Table 1. Primer for RT real-time PCR and RT-PCR[a]

Gene	Direction	Sequence
TNF-α	Forward	5′-GAA CTG GCA GAA GAG GCA CT-3′
	Reverse	5′-AGG GTC TGG GCC ATA GAA CT-3′
TGF-β1	Forward	5′-TGC GCT TGC AGA GAT TAA AA-3′
	Reverse	5′-AGC CCT GTA TTC CGT CTC CT-3′
IFN-γ	Forward	5′-ACT GGC AAA AGG ATG GTG AC-3′
	Reverse	5′-TGA GCT CAT TGA ATG CTT GG-3′
β-Actin	Forward	5′-GAA GTG TGA CGT TGA CAT CCG-3′
	Reverse	5′-TGC TGA TCC ACA TCT GCT GGA-3′
EGFP	Forward	5′-AAG TTC ATC TGC ACC ACC G-3′
	Reverse	5′-TCC TTG AAG AAG ATG GTG CG-3′
FoxP3	Forward	5′-TCT TGC CAA GCT GGA AGA CT-3′
	Reverse	5′-AGC TGA TGC ATG AAG TGT GG-3′

[a]RT-PCR, reverse transcription–PCR; EGFP, enhanced green fluorescence protein.

Study Design

Eight- to 12-wk-old male C57Bl/6J mice (Charles River, Sulzfeld, Germany) were used throughout the studies. Animals were maintained in a virus/antibody-free central animal facility of the Innsbruck Medical University. Accelerated anti-GBM nephritis was induced as described previously (21). In brief, mice were preimmunized subcutaneously with 2 mg/ml rabbit IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) dissolved in incomplete Freund's adjuvant (Sigma, St. Louis, MO) and nonviable desiccated *Mycobacterium tuberculosis* H37a (Difco Laboratories, Detroit, MI). Two days later, one group of mice received 1×10^6 $CD4^+CD25^+$ T cells (Treg), whereas the control group received 1×10^6 $CD4^+CD25^-$ T cells intravenously. In a separate experiment, animals received 1×10^6 Treg or 1×10^6 $CD4^+CD25^-$ T cells (control group) isolated from nephritic animals 7 d after induction of anti-GBM GN. After 3 d, heat-inactivated rabbit anti-mouse GBM antiserum (containing <0.1 EU/ml LPS as determined by Limulus amebocyte assay; Charles River) was injected via the tail vein in a concentration of 5 mg/20 g body wt. Twenty-four-hour urine samples were collected in metabolic cages at days −1, 1, 7, and 14 after induction of disease. At days 1, 7, and 14, a set of animals was killed with CO_2 inhalation.

Urinary Albumin and Urinary and Serum Creatinine Detection

Urinary albumin was determined by a double-sandwich ELISA (Abcam, Cambridge, MA) as reported previously (21). Urinary creatinine was quantified spectrophotometrically using a commercially available kit (Sigma).

Assessment of Glomerular Injury

Formalin-fixed renal tissue was embedded in paraffin, cut in 4-μm sections, and stained with periodic acid Schiff (PAS) for histologic analysis. In all cases, equatorial glomerular cross-sections (minimum of 50 per case) were evaluated as described previously (22).

Sections cut from frozen tissue (4 μm) were used for immunofluorescence or immunoperoxidase staining using standard techniques as described previously (2). For the detection of heterologous IgG deposition, sections were stained by direct immunofluorescence with a FITC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories). For the detection of autologous IgG, FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) was used. Semiquantitative assessment of the glomerular deposition of IgG was

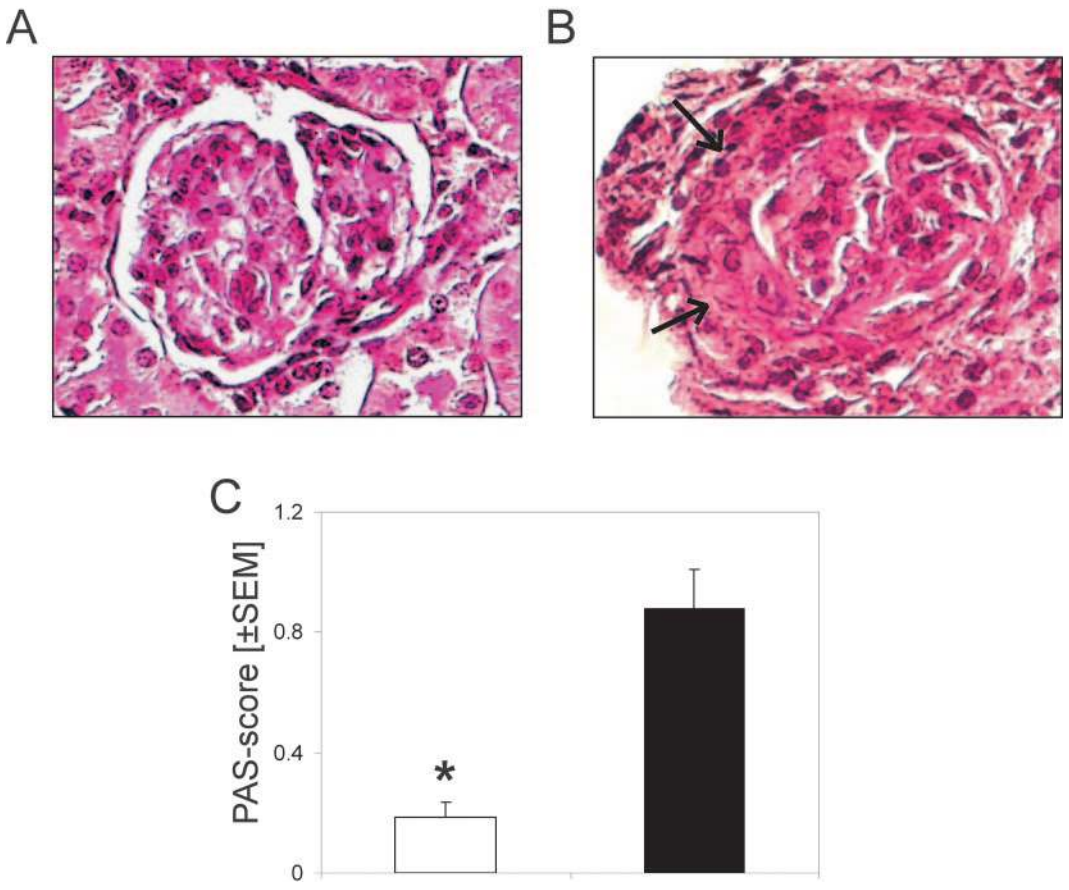

Figure 3. Treg transfer markedly inhibits morphologic changes after induction of GN. Representative periodic acid Schiff (PAS)-stained sections from day 14 are shown. (A) A representative glomerulus from a mouse that received Treg showed only enlargement but no PAS-positive deposits and crescent formation. (B) A representative glomerulus from a mouse that received $CD4^+CD25^-$ control T cells showed enlargement, increased PAS-positive deposits, an increase in mesangial matrix formation, slightly increased numbers of mesangial cells, and global crescent formation (arrows) with a minimum of two layers of epithelial cells. (C) PAS-positive deposits in mice after either Treg (□) or $CD4^+CD25^-$ T cell transfer (■) were quantified as a measure of glomerular injury. Scores are as follows: 0 = no deposits of PAS-positive material; 1 = up to one third; 2 = one third to two thirds, and 3 = more than two thirds of the glomerular cross-section stain positive for PAS (*$P < 0.05$; $n = 7$ per group). Magnification, ×60.

performed by determining the end point positive titer for detection of staining using serial dilutions of each antibody.

The three-layer immunoperoxidase technique was used for the detection of macrophages and T cells in the kidney sections as described previously (21). Macrophages were stained using a rat anti-mouse macrophage antibody (Clone F4/80; Serotec, Oxford, UK), and a semiquantitative scoring system was performed as follows: 0 = 0 to 4 cells stained positive, 1+ = 5 to 10 cells, 2+ = 11 to 50 cells, 3+ = 51 to 200 cells, and 4+ = >200 cells stained positive per low-power field. For the detection of $CD4^+$ T cells, a rat anti-mouse CD4 mAb (clone YTS191.1; Serotec) was used, and for $CD8^+$ T cells, a rat anti-mouse CD8α mAb (clone KT15; Serotec) was used. In all cases, an IgG2a isotype antibody (clone G155–178; Pharmingen, San Diego, CA) served as a negative control. Biotin-conjugated goat anti-rat IgG antibody (Jackson ImmunoResearch Laboratories) was used as a secondary antibody, followed by incubation with an avidin-biotin complex and subsequent development with 0.4% 3-amino-9-ethylcarbazole for 10 min and counterstaining with Gill's Hematoxylin No. 3 (Polysciences Inc., Warrington, PA). Quantification of T cells was done by counting the number of cells in six adjacent high-power fields of renal cortex and medulla.

Reverse Transcription Real-Time PCR and Reverse Transcription–PCR for Enhanced Green Fluorescence Protein and FoxP3

Total RNA was isolated using TRIzol (Sigma) according to a standard protocol. Thereafter, 1 μg of total RNA was reverse transcribed using Omniscript Reverse Transcription Kit (Qiagen, Hilden, Germany) and random primers (Roche, Basel, Switzerland). Real-time PCR was performed on an ABI Prism 7700 (Applied Biosystems, Foster City, CA) using SYBR Green Master Mix (Amersham Biosciences, Piscataway, NJ) and primers (see Table 1) for linear amplification of TNF-α, TGF-β1, IFN-γ, and β-actin (reference gene). Enhanced green fluorescence protein (EGFP)- and FoxP3-positive cells were detected using the primers listed in Table 1. Reverse transcription–PCR (RT-PCR) was carried out using HotStarTaq Polymerase (Qiagen).

Characterization of T Cells from Different Tissues after Cell Transfer

Single-cell suspensions from inguinal lymph nodes, spleens, and kidneys were subsequently stained with antibodies that recognize the following antigens: CD4 (BD Pharmingen); CD25; CD45RB; CD69; CD62L; and CTLA-4 labeled with FITC, PE, or APC (Immunotools, Freiburg, Germany). To determine the percentage of apoptotic/necrotic cells in either cell suspension, we performed propidium iodide staining. The phenotype of T cells was evaluated by flow cytometry (FACSCalibur; Becton Dickinson, San Diego, CA) and analyzed using CellQuest software.

Detection of Cytokine Production from Splenic Cells

Freshly isolated splenocytes (2×10^6 white blood cells/ml) from mice that received an injection of $CD4^+CD25^+$ and $CD4^+CD25^-$ were seeded in 24-well plates and tested individually for cytokine response to LPS (100 ng/ml; Sigma). Supernatants were harvested after 16 h, and TNF-α, IFN-γ, IL-10, and IL-4 were determined using commercially available ELISA kits (Pharmingen) strictly according to the manufacturer's protocol.

Tracking of Carboxy Fluorescein Diacetate Succinimidyl Ester (CFSE)-labeled or EGFP-Transgenic Treg

Single-cell suspensions from inguinal lymph nodes, spleens, livers, and kidneys were prepared from mice that had received either CFSE-labeled $CD4^+CD25^-$ or $CD4^+CD25^+$ T cells. Cells were stained for $CD4^+$ using a PerCP-conjugated mAb (Pharmingen). Subsequently, the percentage of CFSE-labeled $CD4^+$ T cells was determined by flow cytometric analysis using a FACSCalibur (BD, San Diego, CA) and CellQuest software. In another set of experiments, appearance of transferred $CD4^+$ EGFP-positive Treg was determined by flow cytometry.

Statistical Analyses

Data are given as the mean ± SEM. Significances of differences were determined using the *t* test and the Mann-Whitney *U* test (GraphPad-Prism software).

Results

Treg Transfer Attenuates Glomerular Injury and Interstitial Infiltration in Anti-GBM GN

The therapeutic value of Treg transfer for the treatment of autoimmune-mediated kidney diseases was tested in a complement- and T cell–dependent murine model of GN (21). Transfer

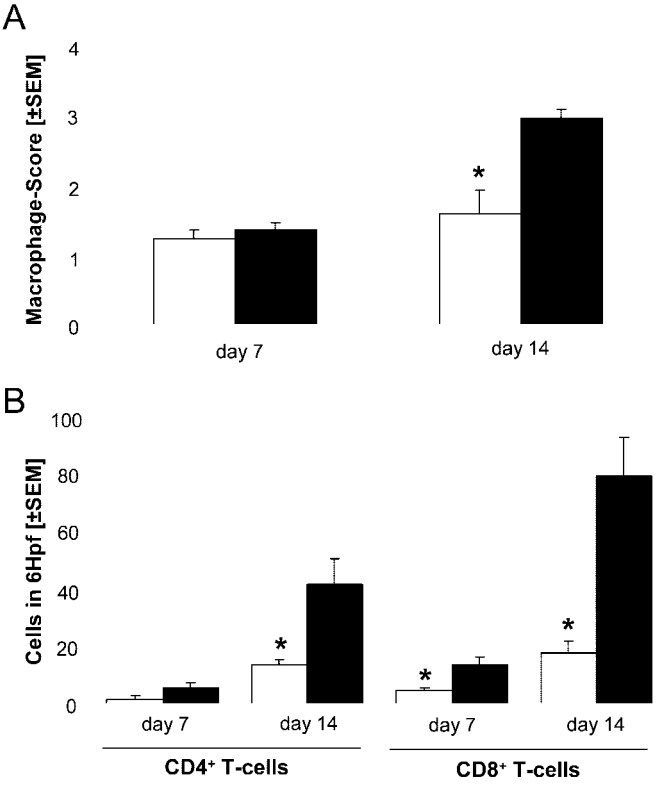

Figure 4. Treg transfer inhibits the infiltration of interstitial macrophages and $CD4^+$ and $CD8^+$ T cells. The infiltration of inflammatory cells was analyzed by immunohistochemistry. Staining for macrophages (A) and $CD4^+$ and $CD8^+$ T cells (B) was performed 7 (n = 14 per group) and 14 d (n = 7 per group) after induction of anti-GBM nephritis in mice that received an injection of Treg (□) or $CD4^+CD25^-$ T cells (■). Interstitial macrophage accumulation was comparable in both groups at day 7 but significantly diminished at day 14 after transfer of Treg. $CD4^+$ T cell infiltration was significantly decreased 14 d after anti-GBM injection, whereas $CD8^+$ T cell infiltration was significantly decreased 7 and 14 d after anti-GBM injection in mice that received an injection of Treg (*$P < 0.05$).

of Treg significantly reduced the development of proteinuria at days 7 and 14 after initiation of anti-GBM GN when compared with animals that received $CD4^+CD25^-$ control cells (Figure 2). Mild to moderate hypercellularity, focal deposition of PAS-positive material, and occasional small crescent formations were observed in mice that received $CD4^+CD25^-$ cells (Figure 3B), which was not seen in mice that received an injection of Treg (Figure 3A). Accordingly, a quantitative scoring system revealed a significant reduction of glomerular PAS deposition in mice that received an injection of Treg as compared with animals that received $CD4^+CD25^-$ T cells (Figure 3C).

Anti-GBM GN is characterized by a marked tubulointerstitial leukocyte infiltration, which primarily comprises T cells and macrophages. We observed a prominent accumulation of macrophages and T cells in the interstitium of mice that were treated with $CD4^+CD25^-$ T cells. Reduction of macrophage and $CD4^+$ T cell infiltration was significant 14 d after initiation of GN in mice that received an injection of Treg (Figure 4). In addition, Treg transfer significantly reduced the infiltration of $CD8^+$ T cells at both time points (Figure 4B).

Treg from Nephritic Animals Have Decreased Immunosuppressive Activity

We next tested the hypothesis that transfer of $CD4^+CD25^+$ cells from nephritic animals might induce the expansion of endogenous Treg, which in turn could lead to enhanced immunosuppression. However, transferring 1×10^6 $CD4^+CD25^+$ cells from nephritic animals revealed not only a markedly lower inhibitory effect when compared with Treg from healthy animals but also a dramatic increase of glomerular damage (Figure 5). Of note, the increase of glomerular damage was even more pronounced in the control group that received $CD4^+CD25^-$ T cells from nephritic animals. This could be because anti-GBM–mediated GN is transferable via T cells (23,24). Increased mortality as a result of uremia in mice that received $CD4^+CD25^+$ or $CD4^+CD25^-$ cells from nephritic mice (25 and 50%, respectively) reflects the severity of disease in those animals, as this phenomenon did not occur in mice that received naïve Treg or $CD4^+CD25^-$ cells from healthy animals. We assume that the $CD4^+CD25^+$ cells that were taken from nephritic animals contain a considerable amount of activated T cells, causing aggravation of glomerular damage. This is underlined by flow cytometric analysis of T cells in the spleens after Treg transfer, showing an increase of $CD4^+CD25^+$ T cells additionally expressing the T cell activation marker CD69 (see Figure 10E and text below). These $CD4^+CD25^+CD69^+$ T cells do not have a regulatory activity but rather function as effector T cells (25).

Humoral Response to Nephritogenic Antibody Deposition in the Glomerulus

Because Treg are known to influence humoral immune response (26), we evaluated the deposition of autologous mouse anti-rabbit IgG on the GBM. Seven and 14 d after induction of disease, deposition of the autologous antibody was comparable in both groups (Figure 6), suggesting that Treg did not affect the generation of an efficient B cell immune response.

Treg Transfer Significantly Reduces Th1 Response in Renal Tissue and Splenocytes from Animals That Received Treg

The anti-GBM GN used in this study is known to be strictly dependent on Th1-like cytokines (27). Treg are known to inhibit primarily Th1 cells (28). Thus, we examined whether Treg transfer modulates the mRNA expression of proinflammatory Th1 cytokines and the profibrotic cytokine TGF-β1 in nephritic kidneys. Real-time PCR revealed a reduced expression of

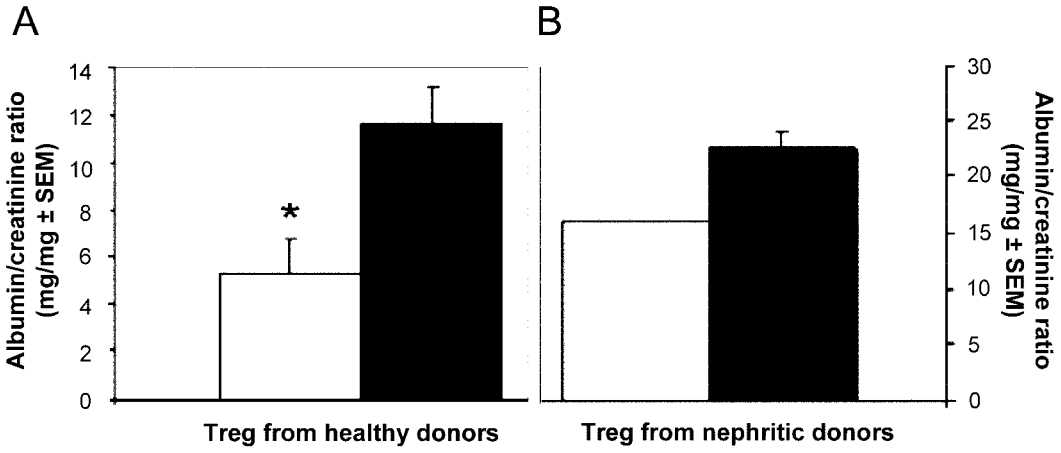

Figure 5. $CD4^+CD25^+$ T cells isolated from nephritic animals have decreased immunosuppressive activity. Fourteen days after induction of anti-GBM GN, proteinuria was evaluated in mice that received $CD4^+CD25^+$ or $CD4^+CD25^-$ cells from nephritic or healthy animals as described in the Materials and Methods section. Urine albumin excretion (in mg) was determined and expressed per milligram of urinary creatinine to standardize for the GFR. (A) Mice that received $CD4^+CD25^-$ T cells from healthy donors showed significant albuminuria (■; $n = 4$), whereas mice after Treg transfer (□; $n = 4$) showed only minimal albuminuria 14 d after induction of anti-GBM GN (*$P < 0.05$). (B) Mice that received $CD4^+CD25^-$ (■; $n = 3$) or $CD4^+CD25^-$ T cells (□; $n = 2$) from nephritic animals developed increased proteinuria 14 d after induction of anti-GBM GN. Note the different scales for the albumin/creatinine ratio in A and B.

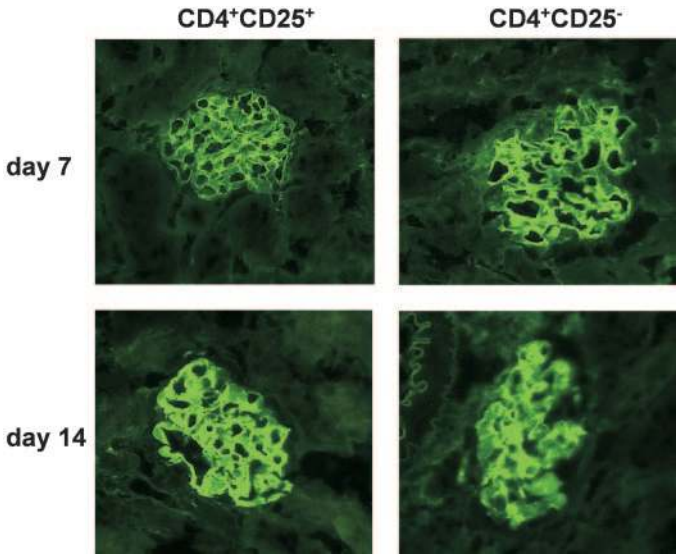

Figure 6. Treg transfer does not affect glomerular deposits of mouse anti-rabbit antibodies. Autologous anti-rabbit antibody (titer 1:1600) was evaluated in renal tissues that were harvested from mice that received an injection of Treg (left) or $CD4^+CD25^-$ T cells (right) 7 and 14 d after anti-GBM injection. A representative glomerulus from each experimental group is shown. Magnification, ×60.

IFN-γ, TNF-α, and TGF-β1 after 7 d, whereas on day 14, the reduction did not reach statistical significance (Figure 7).

To determine the effect of Treg transfer on the production of proinflammatory cytokines, we restimulated the mononuclear cell fraction of splenic cells that were isolated from mice 7 d after induction of anti-GBM GN with LPS, which is known to be an antigen-independent, nonspecific stimulator of mononuclear cells. Splenic cells from the animals that received an injection of Treg produced significantly lower levels of the proinflammatory Th1 cytokines TNF-α and IFN-γ upon stimulation with LPS when compared with splenic cells that were isolated from mice that received an injection of control cells. The secretion of the Th2 cytokines IL-10 and IL-4 was not significantly altered by Treg transfer (Figure 8).

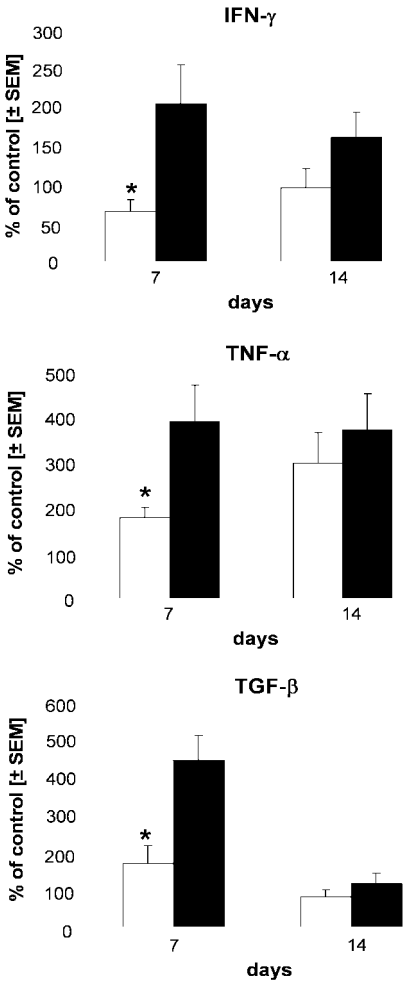

Figure 7. Treg transfer modulates renal mRNA expression of IFN-γ, TNF-α, and TGF-β1. The expression of IFN-γ, TNF-α, and TGF-β1 mRNA was evaluated by real-time PCR of total RNA from kidneys of mice after Treg (□) or $CD4^+CD25^-$ T cell (■) transfer 7 ($n = 14$ per group) and 14 d ($n = 7$ per group) after anti-GBM injection. On day 14, we could not detect significant changes in the expression of the different cytokines, whereas at day 7 IFN-γ, TNF-α, and TGF-β1 mRNA was significantly downregulated in mice that received an injection of Treg (*$P < 0.05$).

Localization of Transferred Treg in GN Animals

In a murine model of experimental inflammatory bowel disease, transferred Treg have been shown to home to lymph nodes and the colon (14). To determine the localization of immunoregulation mediated by Treg in our anti-GBM GN model, we examined the distribution of transferred Treg. Therefore, EGFP-positive Treg were injected into C57Bl/6J wild-type animals, anti-GBM GN was induced, and 1 and 7 d later, RT-PCR for the detection of EGFP in different tissues (spleen, lymph node, kidney, and liver) was performed. After 1 d, we detected only a weak signal for EGFP in secondary lymphoid organs, such as spleen and lymph nodes (data not shown). In contrast, 7 d after induction of anti-GBM GN, a more intense signal for EGFP-positive Treg was found in the secondary lymphoid organs (Figure 9), which might be due to *in vivo* expansion of Treg or their now completed homing progress. It is interesting that transferred Treg were not found in the kidney at day 1 or at day 7 after induction of anti-GBM GN (Figure 9). These data were confirmed by detection of either CFSE- or EGFP-positive cells by flow cytometry in spleen, lymph nodes, liver, but not in the kidney (data not shown). The absence of Treg in the kidney including transferred and endogenous Treg was further confirmed by negative RT-PCR for FoxP3, which is known to be specifically expressed in Treg (Figure 9). Strong signals for FoxP3 were found in secondary lymphoid organs known as reservoirs for Treg. These experiments provide evidence that transferred Treg migrate to and remain, at least during the observed period, in secondary lymphoid organs.

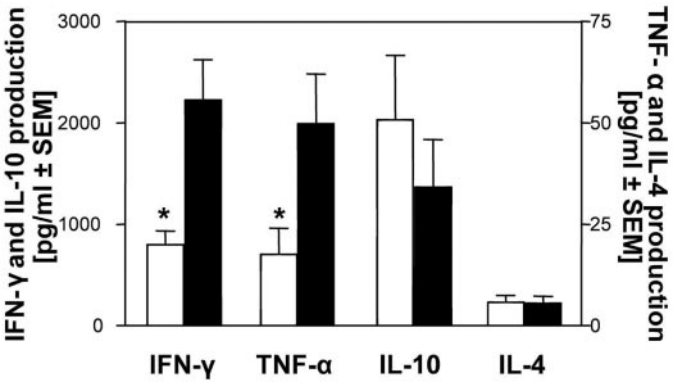

Figure 8. The production of TNF-α and IFN-γ from spleen cell cultures is significantly reduced upon Treg transfer. Splenocytes (2×10^6) from animals that received an injection of either Treg (□) or $CD4^+CD25^-$ T cells (■) were seeded in 24-well plates and stimulated with LPS (100 ng/ml) for 16 h. TNF-α, IFN-γ, IL-10, and IL-4 were determined in supernatants by ELISA (*$P < 0.05$; $n = 4$ per group).

Treg Transfer Primarily Increased Total Treg Content in Peripheral Lymph Nodes

Transfer of Treg led to a significant increase of $CD4^+CD25^+CD69^-CD45RB^{low}$ Treg in peripheral lymph nodes when compared with transfer of $CD4^+CD25^-$ T cells (the $CD4^+CD25^+CD69^-$ fraction is increased by 1.7-fold, the $CD4^+CD25^+CD45RB^{low}$ fraction by 1.6-fold in the Treg-transferred *versus* the $CD4^+CD25^-$ T cell–injected animals; $P < 0.05$ for both cell populations *versus* control cells, $n = 3$ per group; see representative flow cytometric staining in Figure 10A). As already shown by transfer of EGFP-positive Treg as well as by negative FoxP3 RT-PCR, almost all T cells in the kidney expressed CD69 but were negative for CD25, excluding them as Treg infiltrating the kidney (Figure 10B). It is interesting that the relative amount of Treg within the spleen was not increased in mice that received an injection of Treg (Figure 10C).

Discussion

Our current report provides strong evidence that Treg might be of therapeutic value for the treatment of T cell–mediated autoimmune GN. Transfer of Treg significantly improved renal dysfunction in a T cell–mediated model of murine anti-GBM nephritis. The protective effect was not due to inhibition of the initiation of the humoral immune response but rather to inhibited end-organ damage. This is paralleled by a significant reduction of glomerular damage as well as a decrease of T cell and macrophage infiltration. Of note, tracking of transferred Treg revealed a predominant migration of Treg into secondary lymphoid organs, such as spleen and lymph nodes. These findings are of critical importance for the understanding of T cell–mediated immune pathologies of the kidney.

Animal studies provided compelling evidence that, independent of antibody deposition, nephritogenic T cells are sufficient to initiate or amplify inflammatory renal lesions (4,29–31). In humans, the target antigen of anti-GBM nephritis has also been shown to be recognized by both T cells (27,32,33) and B cells (34,35), although only the B cell epitopes have been convincingly characterized. It is well established that $CD4^+CD25^+$ Treg are potent counterparts for autoreactive T cells, thereby maintaining tolerance in rodents and humans (9). In line with this

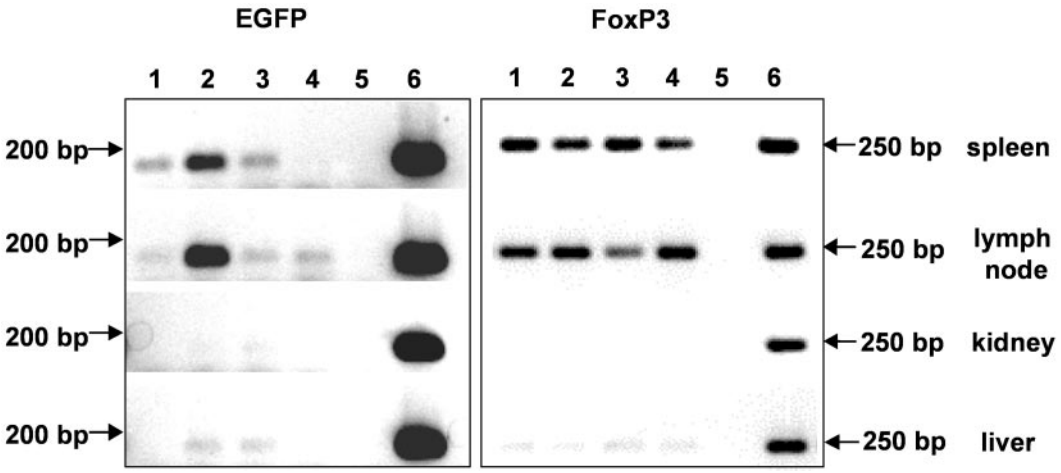

Figure 9. Localization of transferred Treg. For evaluating the tissue distribution of Treg, isolated enhanced green fluorescence protein (EGFP)-positive Treg were transferred into C57Bl/6J mice 1 d before induction of anti-GBM GN (as described in the Materials and Methods section). Spleen, lymph nodes, kidneys, and liver were harvested at day 7 after induction of anti-GBM GN for preparation of total RNA and subsequent reverse transcription–PCR (RT-PCR) for EGFP (left). The EGFP-transgenic donor mouse served as positive control (lane 6). EGFP-positive signals were detected in spleens of three mice and in lymph nodes of all four mice (lanes 1 to 4). In contrast, we detected no signal in the kidney and a weak signal in the liver of mice 7 d after Treg transfer. In addition, we performed RT-PCR for the detection of FoxP3 mRNA expression (right). Isolated Treg served as positive control (lane 6). As expected, FoxP3 signals were detected in spleens and lymph nodes of all four mice (lanes 1 to 4). In contrast, no signal in the kidney and only a slight signal in the liver was observed. As a negative control in EGFP- and FoxP3-PCR, amplification was performed with water instead of a cDNA template (lane 5 in both panels). Equal cDNA template was confirmed by RT-PCR for detection of β-actin (data not shown).

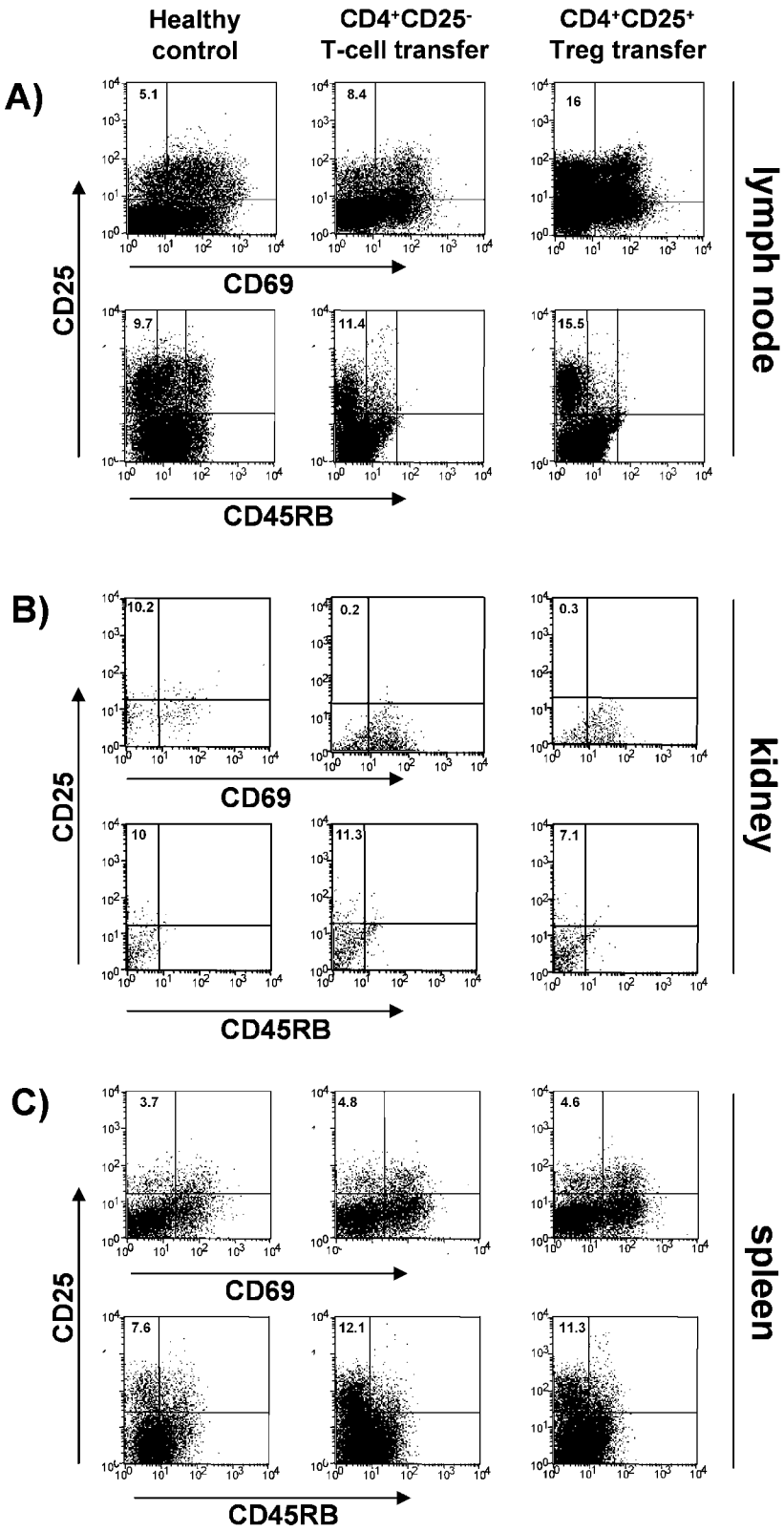

Figure 10. Flow cytometric analysis of CD4-gated T cells in peripheral organs after induction of anti-GBM GN. A representative example of CD25 *versus* CD69 (top) and CD25 *versus* CD45RB expression (bottom) in the lymph node (A), the kidney (B), and the spleen (C) of healthy control animals (left), nephritic mice after $CD4^+CD25^-$ T cell transfer (middle), and animals after Treg transfer (right) is given. A representative example of three animals per group is shown. The relative proportion of $CD4^+CD25^+CD69^-$ and $CD4^+CD25^+CD45RB^{low}$ Treg populations is given in the respective quadrant.

idea, depletion of Treg induces autoimmune diseases in mice. Detailed analysis from humans revealed that patients with autoimmunopathies experience a Treg dysfunction. Accordingly, patients with multiple sclerosis (11), autoimmune-mediated mixed cryoglobulinemia (12), and polyglandular autoimmune syndrome type II (36) display a numerical and functional Treg deficit. Of note, Salama *et al.* (37) recently demonstrated that $CD25^+$ T cells play a critical role in patients who have human Goodpasture syndrome, as they inhibit the autoimmune response directed against the collagen α3(IV)NC1 epitope. These data convincingly demonstrate that under conditions of a decreased immune tolerance to autoantigens, a decrease of Treg number and/or function might promote the development of autoimmune diseases. It is currently not known whether Treg dysfunction is causally involved in autoimmune diseases or is only a secondary phenomenon. However, these observations suggest that restoration of the Treg pool, *i.e.*, by application of Treg, might prevent further tissue damage induced by inhibition of T cell–driven autoimmune pathologies.

In line with this idea, our current report demonstrates the therapeutic efficacy of Treg in experimental murine anti-GBM nephritis. This is shown by a significant reduction of glomerular damage, as well as the inflammatory cellular infiltrate which is significantly impaired by Treg application. Th1 cytokines have been shown to be critical for the development of renal pathology in anti-GBM GN (27,38,39). In line with this observation, we demonstrate a significant reduction of TNF-α and IFN-γ mRNA expression by Treg transfer. It is well characterized that the expression of proinflammatory cytokines is potently suppressed by Treg (9). Of note, TGF-β1 is well known as a key mediator of renal fibrosis in both experimental and human kidney disease, increases the expression of several matrix proteins, and inhibits their proteolytic degradation (reviewed in 40). Therefore, it seems not surprising that TGF-β1 is downregulated in nephritic kidneys of Treg-treated animals compared with controls, despite the predominant role of TGF-β1 in the induction of Treg differentiation by induction of FoxP3 expression (41).

Considering the reduction of the expression of tissue-damaging mediators by Treg application, one could assume that transferred Treg accumulate in the kidneys of nephritic animals, thereby modulating the harmful effects of autoreactive T cells. However, transfer of EGFP-positive Treg isolated from EGFP-transgenic mice into wild-type animals revealed that Treg primarily accumulate in secondary lymphoid organs, with a predominant location in the spleen and lymph nodes. Notably, during the observed time period, EGFP-positive Treg were never found in the kidney but already detected in secondary lymphoid organs on day 1 after transfer. Almost all kidney-infiltrating T cells express the activation marker CD69, excluding them as Treg. Additionally, we were not able to detect mRNA of the Treg-specific transcription factor FoxP3 in total RNA isolated from nephritic kidneys. This observation most likely excludes that transferred Treg initially pass the kidney. This is in contrast to an experimental inflammatory bowel disease model showing that transferred Treg primarily home to the colon and the mesenteric lymph nodes during the first 3 wk after Treg transfer, whereas at later time points they migrated into the spleen (14). In our anti-GBM GN model, it seems that transferred Treg primarily migrate to and expand in lymphoid organs, where the initiation of the immune response takes place. In line with this observation, peripheral lymph nodes contained increased levels of $CD4^+CD25^+CD69^-CD45RB^{low}$ Treg in Treg-transferred animals when compared with mice that received an injection of $CD4^+CD25^-$ control cells. Of note, splenocytes isolated from mice that received an injection of Treg and subsequently were restimulated by LPS showed a decreased production of the Th1 cytokines TNF-α and IFN-γ when compared with application of control cells. However, protection from GN is not mediated by downregulation of Ig production within the B cell compartment of the lymph node or the spleen. This is noteworthy, as Treg are known to suppress the generation of an efficient antibody production (42,43). The exact homing mechanisms after Treg transfer remain to be determined, as they might provide helpful information regarding the modulation of their migratory behavior. A recent report showed that CCL22 might direct Treg migration to ovarian cancer tissue via CCR4 (44). It is conceivable that the modulation of the chemokine receptor repertoire by gene transfer might be a suitable approach for a specific tissue direction of transferred Treg.

In summary, we show for the first time the immunosuppressive activity of $CD4^+CD25^+$ regulatory T cells in experimental GN in mice. We and others recently demonstrated the expandability of human Treg *in vitro*, which resulted in an enhanced Treg-mediated immunosuppressive effect (45,46). These findings together with our presented data from a murine GN model suggest that under conditions of human GN refractory to immunosuppressive agents, the transfer of *in vitro* expanded autologous Treg might represent an additional treatment option.

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See related editorial, "Tipping the Balance in Glomerulonephritis," on pages 1169–1171.