Foxp3-Transduced Polyclonal Regulatory T Cells Protect against Chronic Renal Injury from Adriamycin

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Chronic proteinuric renal injury is a major cause of ESRD. Adriamycin nephropathy is a murine model of chronic proteinuric renal disease whereby chemical injury is followed by immune and structural changes that mimic human disease. Foxp3 is a gene that induces a regulatory T cell (Treg) phenotype. It was hypothesized that Foxp3-transduced Treg could protect against renal injury in Adriamycin nephropathy. CD4+ T cells were transduced with either a Foxp3-containing retrovirus or a control retrovirus. Foxp3-transduced T cells had a regulatory phenotype by functional and phenotypic assays. Adoptive transfer of Foxp3-transduced T cells protected against renal injury. Urinary protein excretion and serum creatinine were reduced (P < 0.05), and there was significantly less glomerulosclerosis, tubular damage, and interstitial infiltrates (P < 0.01). It is concluded that Foxp3-transduced Treg cells may have a therapeutic role in protecting against immune injury and disease progression in chronic proteinuric renal disease.

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AN are nephrotic syndrome, focal glomerular sclerosis, tubular injury, and interstitial compartment expansion with mononuclear cell infiltrates that are composed largely of macrophages and T cells (21–24). Initial studies showed that depletion of CD8 T cells ameliorated AN and that depletion of CD4 T cells worsened disease (22,25). However, in immune-deficient SCID mice, AN is more severe than in BALB/c mice with lower doses of ADR and is still accompanied with heavy macrophage infiltrate, suggesting that the cognate immune system contains a subset of protective cells (unpublished data). The CD4+CD25+ subset of T cells therefore is a potential candidate for this role. We therefore wished to examine the effect of Treg on AN.

Treg were created by retroviral gene transfer of Foxp3 into CD4+ T cells. We evaluated the transduced T cells for regulatory markers and tested their ability to inhibit T cell activation in vivo. We then tested the effect of the Foxp3-transduced T cells on AN. We further depleted Treg using anti-CD25 antibodies. The results show that Foxp3-transduced CD4+ Treg can protect against renal functional and structural injury in vivo in this murine model of chronic proteinuric renal disease, whereas depletion of CD4+CD25+ T cells exacerbates AN.

Materials and Methods

Mice

Male BALB/c mice were obtained from the ARC (Perth, Australia). All mice were maintained free of pathogens in the Westmead Hospital animal house. Eight-week-old mice that weighed 20 to 22 g were used in all groups.

Cell Culture and Retroviral Transduction

The cell lines that were used include NIH/3T3 and packaging cell line EcoPack2-293 (BD Biosciences, North Ryde, Australia). The cell lines were cultured in DMEM (Invitrogen, Carlsbad, CA). Foxp3/MIGR1 and MIGR1 vectors were provided by Dr. Horii (11) and Dr. Pear (26). The retroviral vectors Foxp3/MIGR1 and MIGR1 were transduced into the packaging cell line EcoPack2-293 using calcium phosphate, and the viral supernatant was collected (27). The viral titer was 10⁴ cfu/ml. Mouse CD4+ T cells were isolated from BALB/c mouse spleens by MACS CD4+ MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and stimulated by anti-CD3 mAb (5 µg/ml; BD Biosciences) and rIL-2 (100 IU/ml) for 24 h and then were infected with either Foxp3/MIGR1 or MIGR1 viral supernatant using retronectin (Edward Keller, Hallam, Australia) and Vivaspin (Sartorins, East Oakleigh, Australia). GFP-positive cells then were sorted by flow cytometry at day 7 after transduction (BD FACS Vantage SE).

Adoptive Transfer of Foxp3-Transduced Cells and Induction of AN

After sorting, 1 × 10⁶ MIGR1/Foxp3- or MIGR1-transduced cells were injected into the tail vein of each BALB/c mouse. Mice were divided into four groups (five mice in each group): (1) the ADR group (received ADR only), (2) the ADR+Foxp3 group, (3) ADR+MIGR1 group, and (4) the control group. Both transduced groups were injected 1 wk before injection with ADR. ADR (doxorubicin hydrochloride; Pharmacia & Upjohn Pty Ltd., Perth, Australia) was injected via the tail vein of each nonanesthetized mouse (9.6 mg/kg). Mice in the control group were treated with saline only. Body weights were measured daily until day 28 after ADR. Blood, spleen, and kidney samples were obtained from mice in each group.

Depletion of CD4+CD25+ Cells In Vivo for AN Model

A purified rat anti-mouse CD25 monoclonal IgG1 antibody (PC61; Bio Express, West Lebanon, NH) was used to deplete mouse CD4+CD25+ cells in vivo. Five mice received an intravenous injection of 0.25 mg of PC61 2 d before ADR injection. Another dose of 0.25 mg of PC61 was injected intravenously 2 d after ADR injection. The efficacy of CD25 depletion was confirmed by flow cytometry analysis, using PE-conjugated anti-mouse CD25 and FITC-conjugated anti-mouse CD4 (ADR + PC61).

Renal Function

Renal function was assessed by measurement of urine protein and creatinine and of blood albumin and creatinine. Urine was collected from each mouse for 16 h before being killed. Urine volume, protein, and creatinine were measured. Blood samples for serum albumin and creatinine were obtained by cardiac puncture at day 28. Creatinine in serum and urine was measured by the CREA method, serum albumin was analyzed by the ALB method, and total urine protein was measured by the UCFP method on the Dimension Clinical Chemistry System (Dade Behring Ltd., Deerfield, IL).

Proliferation Assays

Ninety-six-well flat-bottom plates were coated with anti-CD3 mAb (5 µg/ml; BD Biosciences). Sorted CD4+CD25+ cells (2.5 × 10⁴ per well) were cultured alone or in the presence of a 1:1 ratio of transduced GFP-expressing cells (sorted from cells that were transduced with Foxp3 or MIGR1), or sorted CD4+CD25+ cells were cultured in a final volume of 200 µl of complete medium at 37°C for 72 h. Titration to a ratio of targets:suppressor of 1:0.5 was also performed. During the last 16 h of culture, cells were pulsed with 3H-thymidine (1 µCi/well; MP Biomedicals, Seven Hills, Australia). Cells were assessed for thymidine incorporation in a Microbeta counter (Wallac Oy 1450 MicroBeta; Wallac, Melbourne, Australia).

RNA Isolation and Real-Time PCR

RNA was isolated from mouse lymphocytes, kidney, spleen, and lymph node using TRIZOL Reagent (Invitrogen, Life Technologies, Mount Waverly, Australia). The total amount of RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen, Life Technologies) and random primer (Promega, Madison, WI). Foxp3 mRNA levels were quantified by real-time PCR using the ABI PRISM 7700 (PE Applied Biosystems, Foster City, CA), and cDNA samples were subjected to real-time quantitative PCR analyses using primers and an internal fluorescence probe specific for Foxp3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). PCR mixture contained 0.3 µm primers and 0.05 µm TaqMan probe and was cycled for 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. The primers and TaqMan probe sequences for real-time PCR were as follows: Foxp3 primers 5′-TTGGCCAGCCGCGTATCTT-3′ and 5′-TGGCTCTCTCCAGAGAGAAAGTG-3′, Fox3 probe 6FAMCAGCTGCTGCACGMCMBNFOQ, GAPDH primers 5′-TGGACCCACCAACTGCTTACG-3′ and 5′-GAAGACCCGATGCCAGTGA-3′, and GAPDH probe VICCCCT-GCCAAAGTCATCCATGAACCT-TAMRA. The normalized value for Foxp3 mRNA expression was calculated as the relative quantity of Foxp3 divided by the relative quantity of GAPDH. Triplicate samples were run.

Reverse Transcription–PCR Conditions and Primers

The expression of TGF-β, CTLA-4, CD103, and GITR was measured by reverse transcription–PCR (RT-PCR). RNA isolation and cDNA amplification were performed as above. cDNA was subjected to nested PCR amplification.
using external and internal primer couples, and β-actin was used as a control. The PCR conditions were 95°C for 5 min (1 cycle); 95°C for 45 s, 60°C for 45 s, and 72°C for 1 min (35 cycles) with the final cycle at 72°C for 7 min. The following primers were used: CTLA-4, 5'-TGGGGGCAATTGAGGCTTCTA-3' and 5'-TGGGGATACGTTGTAAGGGGCTC-3'; GITR, 5'-AACGGAATTGCGAACCACACT-3' and 5'-TGGTTGGAGAAGGAGGAGGAAC-3'; TGF-β, 5'-TGACGGAAGAACCATCCTGATGAAA-3' and 5'-TGGAGAGGTGTTCAAGGCTCTTGCT-3'; CD103, 5'-CTGGAGAAGGACGACAGT-3' and 5'-CTGGAGTAAAGGAGCTTAGAT-3'; and β-actin, 5'-TGGTTCAGAGGACTCTCTATG-3' and 5'-CAGGCAAGCTGCTCTTCT-3'. The PCR products were run on 1.5% agarose gels and visualized under ultraviolet light using gel-doc 1000 (Bio-Rad, Hercules, CA).

Flow Cytometric Analysis

Antibodies that were used for flow cytometry included FITC-conjugated anti-mouse CD25, phycoerythrin (PE)-conjugated anti-mouse CD3, and PE-conjugated anti-mouse CD4 (BD Bioscience). Foxp3 intracellular staining was performed by using PE anti-mouse Foxp3 Staining Set (clone FJK-16s; eBioscience, San Diego, CA) following the manufacturer's instructions. All samples were analyzed on a FACSscan analyzer (Becton Dickinson, Mountain View, CA). CellQuest software was used for analysis (Becton Dickinson, North Ryde, Australia).

Histology and Morphometric Evaluation

The kidneys were removed rapidly on day 28. Sagittal slices of renal tissue were fixed in neutral buffered formalin at room temperature for 24 h and embedded in paraffin for evaluation of pathology. Five-micrometer slices were stained with periodic acid-Schiff reagent and assessed by light microscopy. The remaining cortex of the same kidney was snap-frozen in liquid nitrogen and used for RNA and immunohistochemistry. In each biopsy, a semiquantitative score from two blinded, trained observers was used to evaluate the degree of renal injury, and was graded on a scale of 0 to 4 (28).

Immunohistochemistry

Immunohistochemical staining was performed for CD4+ T cells, CD8+ T cells, and macrophages. Primary antibodies that were used in immunohistochemistry were rat anti-mouse L3T4 (Sigma, Balcatta, Australia) for CD4+, rat anti-mouse Ly-2 (PharMingen, Inc., Sydney, Australia) for CD8+, and rat anti-mouse Mac-3 (PharMingen, Inc.) for macrophages. The secondary antibody was a biotinylated rabbit anti-rat Ig (Dako Corp., Carpinteria, CA). A section from each kidney or spleen was placed in OCT (Sakura Finetek Inc., Torrance, CA). Six-micrometer sections were cut, dried, and fixed in cold acetone. Endogenous peroxidase activity was blocked by incubating sections for 15 min in 0.3% (vol/vol) H2O2 solution. Endogenous avidin binding activity was blocked by incubating the sections with Biotin Blocking System (Dako). For control sections, normal rat Ig was used. Sections were incubated with secondary antibodies, 3,3-diaminobenzidine substrate-chromogen solution (Dako) was applied, and then sections were washed. Slides were counterstained with hematoxylin (Sigma). For assessment of interstitial infiltration, positively stained cells that were located in interstitium only were counted from five random cortical fields (Magnification, ×400). Glomerular macrophage infiltration was evaluated as the number of macrophages per 10 glomerular cross-sections.

Assessment of Number of Adoptively Transferred Cells In Vivo

CD4+ T cells were purified by MACS CD4+ MicroBeads (Miltenyi Biotec) from splenocytes of B6STL-ly5.1 mice, and 1 × 106 CD4+ T cells were transferred by tail-vein injection into congenic C57Bl/6 mice (Ly5.2). Twenty-four hours after transfer, peripheral blood was assessed for relative expression of Ly5.1 CD4+ T lymphocytes by flow cytometry using FITC-conjugated anti-CD4 and PE-conjugated anti-Ly5.1.

Semiquantitative RT-PCR for GFP Expression

Semiquantitative RT-PCR for GFP expression was performed on tissues from each experimental group. The primers that were used to amplify the GFP sequences (Invitrogen) were 5’-CCTGAAGTTCATCTCGACACCACC-3’ and 5’-CTGTGCTGTAGGTGTCCGGACG-3’. Amplification conditions were 95°C for 5 min, followed by 40 cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 10 min. β-Actin primers served as the internal control. The sequences used are described above, and PCR conditions were the same as for GFP, except that the reaction was run for 30 cycles. RT-PCR fragments of GFP and β-actin were electrophoresed on a 2% agarose gel. Signals were quantified by Alphalmager analysis system (Alpha Innotech, Quantum Scientific, Lane Cove, Australia).

Statistical Analyses

Statistical analysis was performed by one-way ANOVA for multiple comparisons. Results are expressed as the group mean ± SD. Two group differences were analyzed by t test. Two-tailed P < 0.05 was considered statistically significant.

Results

Retroviral Transduction of Foxp3

Retroviral vectors that expressed Foxp3 and GFP were transfected into the packaging cell line EcoPack2-293, and viral supernatants were transduced into mouse CD4+ T cells. Retroviral transduction led to expression of GFP/Foxp3 in NIH/3T3 cell line and also in primary murine CD4 T cells (Figure 2A). EcoPack2-293, NIH/3T3 cell lines, and T cells that were transduced by either retroviral vectors expressed GFP. One week after transduction, 30% of Foxp3- and 50% of the control MIGR1-transduced T cells showed GFP expression by flow cytometry (Figure 1B).

Transduction of Foxp3 Induces a Treg Phenotype

We examined the expression of Foxp3 mRNA by RT-PCR using Foxp3-specific primers and β-actin as control. Sorted CD4+CD25+ cells showed expression of Foxp3, whereas CD4+CD25− cells did not (Figure 2A). The Foxp3-transduced NIH/3T3 cell lines showed strong Foxp3 expression, whereas cells that were transduced with control retroviral vector showed no Foxp3 expression (Figure 2B). One week after Foxp3 transduction, GFP-positive cells were sorted further by flow cytometry. After sorting, Foxp3-transduced cells had high levels of expression of Foxp3, TGF-β, and CTLA-4 and lower expression of CD103 by RT-PCR (Figure 2C). Control vector (MIGR1)-transduced cells showed no expression of these Treg markers. GTR was found in all T cells possibly secondary to the activation step above.

Foxp3 mRNA expression was assessed in transduced and
control CD4+ T cell subsets. cDNA samples were subjected to real-time quantitative PCR analyses using primers and internal fluorescence probe specific for Foxp3 or GAPDH. Our results showed that Foxp3 mRNA level in CD4+/H11001CD25+ cells was 200-fold more abundant than in CD4+/H11001CD25− cells, and transduced Foxp3 T cells showed >200-fold higher expression of Foxp3 than MIGR1-transduced T cells (Figure 2D).

**Foxp3-Transduced T Cells Inhibit CD4+/CD25− Cell Proliferation In Vitro**

Foxp3-transduced cells specifically inhibited proliferation of freshly prepared CD4+/CD25− responder T cells when stimulated with anti-CD3 mAb. The proliferation of CD4+/CD25− responder T cells was inhibited by freshly sorted CD4+/CD25+ T cells (P < 0.05) as expected, and the Foxp3-transduced cells were able to inhibit proliferation (P < 0.02). In contrast, MIGR1-transduced CD4 T cells did not show any suppressive activity (P > 0.05; Figure 3A). Titration of the suppressor CD4+/CD25+ T cells or the Foxp3-transduced CD4+ T cells had a suppressive effect at a ratio of 1:0.5 (Figure 3B).

**Foxp3-Transduced T Cells Protected against Renal Damage Induced by ADR**

All ADR-treated mice developed renal injury characterized by proteinuria, tubular cell atrophy and tubular dilation, focal segmental glomerulosclerosis, and interstitial expansion with infiltration of mononuclear cells. In the ADR group, tubules
showed a decrease in height of tubular epithelial cells, vacuolization, and a moderate interstitial monocyte infiltration. Glomeruli were reduced in size with severe vacuolization and the expansion of the mesangium under periodic acid-Schiff staining (Figure 4). Similar morphologic changes were observed in the ADR/H11001MIGR1 group. Light microscopic examination revealed that glomerular surface area was significantly diminished in both groups of mice, where the glomeruli were shrunken because of glomerulosclerosis. However, there was only mild damage of the glomeruli and tubules in ADR/H11001Foxp3 group, compared with the ADR and ADR/H11001MIGR1 groups (Figure 4). Morphometric analysis showed significantly less damage in glomeruli and tubules in the ADR/Foxp3 group, compared with the ADR and the ADR+MIGR1 groups ($P < 0.01$, one-way ANOVA tests), as well as reduced interstitial monocyte infiltration in ADR+Fopx3 group, compared with other two groups ($P < 0.05$, one-way ANOVA tests; Figure 5A).

A dense mononuclear cell infiltrate, consisting of macrophages and CD8+ and CD4+ T cells, was found in the interstitium in the ADR and ADR+MIGR1 groups of mice (Figure 6). There was significantly less macrophage infiltration in the ADR/Foxp3 group than in the other ADR-treated groups of mice ($P < 0.01$).

Renal function was measured in each animal at the end of experiment. Urinary protein excretion was significantly reduced in ADR mice that were treated with Foxp3-transduced cells (ADR/H11001Foxp3), compared with those that received ADR and those that received control vector (ADR/H11001MIGR1; $P < 0.001$; Figure 5B), whereas proteinuria was worse in mice that were depleted of Treg (ADR+PC61). Serum creatinine was also significantly lower in the ADR+Fopx3 group than in the ADR and ADR+MIGR1 groups of mice ($P < 0.01$) but was not different between ADR and ADR+PC61 (Figure 5C). Furthermore, the ADR+Fopx3 group did not experience the weight loss seen in the other ADR-treated groups ($P < 0.001$; Figure 5D) after PC61 depletion of CD25 T cells (Figure 5E).

**Relative Number of Transferred CD4 T Cells**

For assessment of the relative number of transferred CD4 T cells, $1 \times 10^6$ CD4 T cells that were purified from a congenic
Ly5.1-expressing mouse were transferred by tail-vein injection into a C57Bl/6 mouse of similar size as the experimental mice. At 24 h, the adoptively transferred cells composed 5.7% of the total CD4+/H11001 lymphocyte population (Figure 7A).

**Location of the Transferred Transduced T Cells**

Both transduced groups of CD4 T cells contained GFP. We therefore used RT-PCR for GFP and identified both Foxp3 and control MIGR1-transduced T cells in the spleen, draining lymph nodes, and kidneys of the AN mice (Figure 7B). We further assessed Foxp3 levels in the kidney and lymph nodes by real-time PCR to demonstrate high levels of Foxp3 within both the lymph nodes and the affected kidneys of mice that received Foxp3-transduced cells (Figure 7C).

**Assessment of Foxp3 Expression in AN**

Mononuclear cells were obtained from spleen, lymph node, and kidney of wild-type (WT) and AN mice and stained with
CD4, CD25, and intracellular Foxp3. Treg as defined by Foxp3 expression composed 12 to 19% of CD4 T cells in WT spleen and lymph node but not in CD8 T cells (Figure 8A). No CD4 T cells were detected in WT kidney. Similar proportions of Foxp3-containing CD4 cells (11 to 17%) were found in AN spleen, lymph node, and kidney (Figure 8B). CD25 was expressed on a majority of Foxp3-containing CD4 T cells but was lower on cells within the kidney (Figure 8C).

**Discussion**

Treg that were generated from naïve CD4+ T cells by retroviral gene transfer of Foxp3 were highly effective in the treatment of AN. The Foxp3-transduced cells expressed regulatory molecules, including CTLA-4, GITR, CD103, and TGF-β. They demonstrated suppressive function in *in vitro* assays. Foxp3 transduction alone was sufficient to induce CD4+CD25+ Treg function in naïve T cells. The reduction in both Foxp3 expression of MIGR1-transduced T cells relative to CD4+ T cells and TGF-β expression of MIGR1-transduced T cells relative to CD4+CD25− T cells is likely a function of the activation and culture conditions that are required for retroviral transduction. TGF-β is a requirement for Treg survival, and its absence in the culture may explain the results suggestive of a loss of a Treg subset in the MIGR1-transduced controls (29). In addition, the effect of CD25 depletion in exacerbating AN may be tempered by Treg that contain Foxp3 but do not express CD25. Tissue-based Foxp3-expressing Treg have a larger proportion of cells that do not express CD25. Therefore, the depletion of CD25, although showing some effect, may not completely deplete all Treg, particularly those in the tissues (16).

Array studies of Foxp3-expressing CD4+ T cells suggest that they express a broad range of chemokine receptors CCR2, CCR4, and CCR8, and functional studies show that Treg protection against graft-versus-host disease is not effective in the absence of CCR5 (16,30). We showed previously that a range of chemokines, including CCL2, are expressed in the AN kidney and may help attract Treg to the kidney (31).

In neither chronic proteinuric renal disease in humans nor murine AN has a specific antigen been defined. Therefore, the
strategy of pathogenic TCR antigen–specific T cells with regulatory properties is not currently an option in chronic proteinuric renal disease. However, the general effects of Foxp3-transduced T cells may limit their general use because of nonspecific effects in areas such as cancer immunosurveillance and infection.

The ability of polyclonal Treg to protect against renal injury raises a number of questions. Models of disease using TCR specific for pathogenic antigens seem more potent in treating disease (12,15). The strength of regulation seems to be enhanced by Treg that are specific for pathogenic antigens (32). However, in addition to their antigen specificity, Treg seem to have a more general immunosuppressive effect. Thus, although the predominant mode of action of Treg studied has been their effects on the cognate immune system, the data on the effect of Treg on the innate immune system may explain the effect of a polyclonal rather than TCR-directed population of CD4 Treg in AN. In a Helicobacter disease model in RAG-1−/− mice that were deficient in T and B cells, Treg ameliorated disease (33). Similarly, in a murine model of burn injury, there was evidence of CD4+ Treg suppressing innate immune activation through TLR2 and TLR4 (34). Data suggesting this have also been shown in vitro, where Treg impaired the maturation and antigen presentation of dendritic cells (35). This concurs with our previous data that CD4 depletion significantly aggravated AN in BALB/c mice with increased accumulation of macrophages (25).

This idea of regulation’s occurring against T cells that bear specific TCR and regulation’s occurring through the recognition of other activating markers such as CD25 or CD69 was
addressed recently (36). The effects in our model suggest either a non–TCR-restricted effect or a direct regulation of innate effectors such as dendritic cells or macrophages.

Adoptive cell therapies are beginning to enter clinical application. The use of cytotoxic T cells in a T-cell–depleted environment has reached clinical application in the treatment of malignancy, whereas virus-specific T cells have been used to treat cytomegalovirus and other viral diseases (37–41). Therapeutic cell therapies with regulatory T cells are still in their infancy. Depletion and transfer strategies in mice have been used successfully to demonstrate the effect of this regulatory subset on disease progression (1,42). In addition, the TCR specificity that is required for regulation may further limit the effectiveness of directly isolated Treg (12,15). A number of strategies to overcome this have been proposed, including ex vivo expansion of Treg using cytokines such as TGF-β, IL-2, and IL-15 (43–45).

Of particular interest is the induction of specific regulation by forced expression of the regulatory gene Foxp3. Our studies show that previous administration of Foxp3 Treg can limit renal injury either by inhibiting pathogenic T cells or possibly by limiting innate immune injury and raise the possibility of these as a therapeutic strategy to protect against renal injury.

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