

IL-2/IL-2Ab Complexes Induce Regulatory T Cell Expansion and Protect against Proteinuric CKD

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ABSTRACT

Regulatory T cells (Tregs) help protect against autoimmune renal injury. The use of agonist antibodies and antibody/cytokine combinations to expand Tregs *in vivo* may have therapeutic potential for renal disease. Here, we investigated the effects of administering IL-2/IL-2Ab complexes in mice with adriamycin nephropathy, a model of proteinuric kidney disease that resembles human focal segmental glomerulosclerosis. Injecting IL-2/IL-2Ab complexes before or, to a lesser extent, after induction of disease promoted expansion of Tregs. Furthermore, administration of this complex was renoprotective, evidenced by improved renal function, maintenance of body weight, less histologic injury, and reduced inflammation. IL-2/IL-2Ab reduced serum IL-6 and renal expression of IL-6 and IL-17 but enhanced expression of IL-10 and *Foxp3* in the spleen. *In vitro*, the addition of IL-2/IL-2Ab complexes induced rapid STAT-5 phosphorylation in CD4 T cells. In summary, these data suggest that inducing the expansion of Tregs by administering IL-2/IL-2Ab complexes is a possible strategy to treat renal disease.

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The key role of regulatory T cells (Tregs) has been identified in immune homeostasis and in suppressing unwanted inflammatory responses toward self-antigens.^{1,2} In a variety of autoimmune diseases, there are reduced numbers of Tregs predisposing to disease.³ In renal disease we and others have shown that the *Foxp3*-expressing subset of Tregs protect against renal disease, that removal of Tregs exacerbates disease, and that T cells transduced with *Foxp3* to become Tregs are protective.⁴ The protection occurs in both immunodeficient (severe combined immunodeficiency) and immune-intact mice (BALB/c), suggesting that Tregs, in addition to suppressing cognate immune cells, can act on the innate immune response and

potentially directly on injured renal tissue.^{4,5} The regulatory and production difficulties of *ex vivo* expansion of Tregs for therapeutic use in humans make *in vivo* expansion a potentially attractive option. However, enthusiasm for this has been tempered by the experience with TGN1412, the CD28 agonist that led to Treg expansion in rat models and protection against crescentic GN but caused pathologic T cell activation in human trials.^{6–9}

IL-2 acts through the high-affinity IL-2 receptor composed of α , β , and γ subunits. It activates CD4⁺ and CD8⁺ T cells and Tregs.¹⁰ It can also activate cells with low-affinity $\beta\gamma$ IL-2Rs, such as memory CD8⁺ and natural killer (NK)

cells.^{11,12} Tregs are exquisitely dependent on IL-2 for survival, and Tregs can be eliminated by neutralizing anti-IL-2 mAb. The potency of IL-2 appears to be enhanced when it is complexed with certain antibodies. Of particular interest is the substantial selective Treg expansion in mice after injection of IL-2 complexed to a specific IL-2 mAb clone (JES6-1).^{13,14} This induces a temporary three- to four-fold increase in Treg numbers *in vivo*, potentially through enhanced binding to CD25, and is protective in experimental models of allergy, experimental allergic encephalitis (EAE), and islet transplantation.^{13–15} This is in comparison to S4B6 anti-IL-2 mAb, which blocks CD25 binding but enhances IL-2R β binding and thereby leads to CD8 rather than Treg activation.¹⁶

In this study, we used IL-2 complexed to JES6-1 IL-2 mAb to treat adriamycin nephropathy (AN), a model of chronic proteinuric renal disease, before and after AN induction with adriamycin.^{4,5} We

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found that mice treated with IL-2/IL-2Ab complex before and after adriamycin administration showed significant reduction in renal injury. This may allow Treg therapy that uses *in vivo* expansion rather than *ex vivo* cellular therapy to protect against kidney disease.

AN is a murine model of chronic proteinuric renal disease that resembles human focal segmental glomerulosclerosis. We compared pretreatment and post-treatment findings using the IL-2/IL-2Ab complex in AN mice. We used a clinically designed protocol with a short three-dose regimen before AN and a longer post-AN six-dose regimen to test efficacy and potential therapeutic utility. Mice were injected with the IL-2/IL-2Ab complex. Splenocytes of treated animals were analyzed by flow cytometry for CD4+CD25+*Foxp3*+ Tregs. Five to 6 days after IL-2/IL-2Ab complex injection, spleen size increased (Figure 1E) and CD4+ *Foxp3*+ cells increased from a mean \pm SD of $14 \pm 3\%$ to $47 \pm 4\%$ of the splenic CD4+ T cells ($n=7$), and CD4+CD25+*Foxp3*+ T cells increased from $9 \pm 2\%$ to $46 \pm 3\%$ of the splenic CD4+ T cells ($n=7$) (Figure 1A). Immunohistochemical sections from mice injected with IL-2/IL-2Ab complex showed increased numbers of *Foxp3*+ Treg cells in the spleen at days 5–6 after IL-2/IL-2Ab complex treatment (Figure 1F). This expansion is similar to that reported previously using C57Bl/6 mice.¹³

At day 6 after IL-2 complex stimulation *in vivo*, other splenic subsets were assessed, including CD4, CD8, and NK cell numbers. CD4+ T cells (increased from 57% to 64%) showed greater expansion than did CD8+ T cells (decreased from 42% to 34%) and NK cells (decreased from 54% to 14%). Activation as assessed by loss of CD62L expression was reduced in NK cells in IL-2 complex-treated mice, whereas CD8 T cells showed an increase in the activated CD62L lo proportion (Figure 1, B–D).

In the AN group, mice had severe renal injury, characterized by heavy proteinuria, elevated serum creatinine, and body weight loss. Histologic examination revealed tubular cell atrophy, significant glomerular injury, and a moderate interstitial

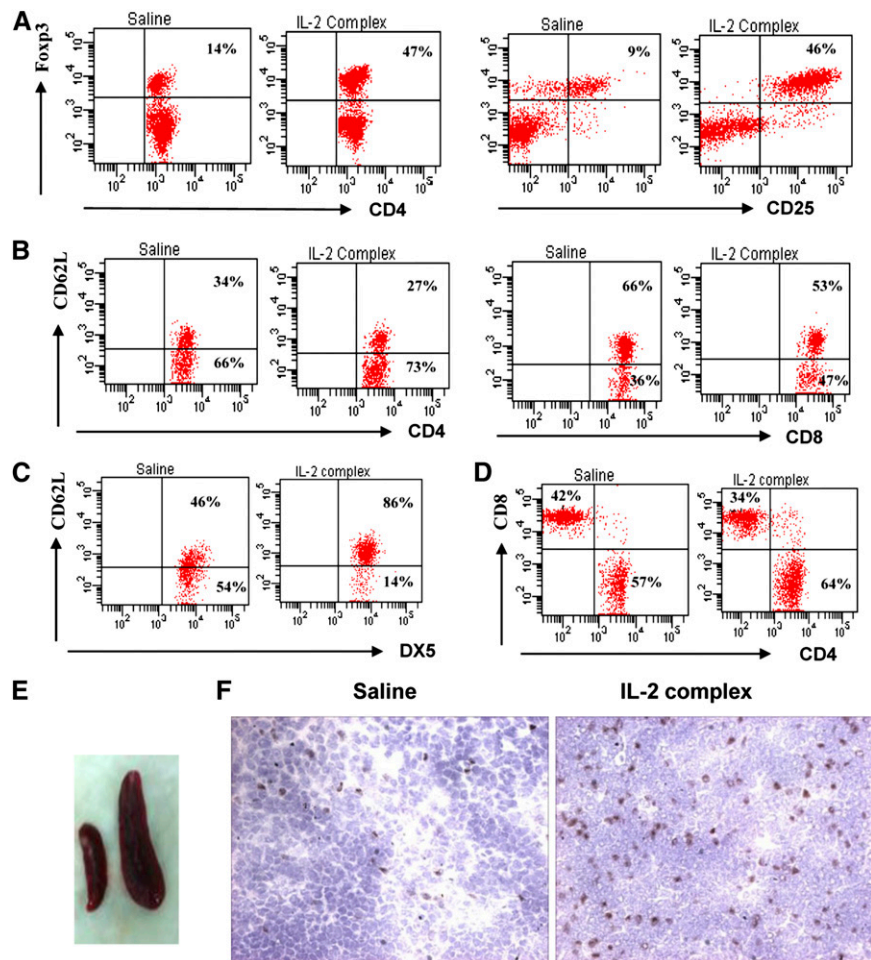


Figure 1. IL-2/IL-2Ab complex induces *Foxp3*+ Treg expansion *in vivo*. Mice received IL-2/IL-2Ab complex or saline intraperitoneally daily for 3 days. CD4+CD25+*Foxp3*+ Tregs from splenocytes were stained and analyzed by flow cytometry at day 6. CD4+ *Foxp3*+ cells increased from 14% to 47%. CD4+CD25+*Foxp3*+ T cells increased from 9% to 46% (A). CD4+ T cell expansion was greater (57% increased to 64%) than CD8+ T cells (42% decreased to 34%) and NK cells (54% decreased to 14%) (B and D). NK cells were less activated with higher CD62L expression, whereas there was a slight increase in activated CD8+ T cells (CD62L lo) (C). There was an increase in spleen size (E) and increased numbers of *Foxp3*+ T cells in spleen. (F) Immunohistochemical staining 5 days after IL-2/IL-2Ab complex treatment. Data are representative of three independent experiments ($n=5-7$). Original magnification, $\times 400$.

infiltrate of inflammatory cells. Mice in the pre-IL-2 group had only mild damage of the glomeruli and tubules ($P<0.01$; Figure 2A, Figure 3), lower serum creatinine, and less proteinuria ($P<0.01$, $P<0.05$; Figure 2, B–D) and better maintenance of body weight ($P<0.05$; Figure 2E). Mice in the post-IL-2 group also showed reduced renal injury, with less glomerular and tubular damage and interstitial infiltration, but the complex was less protective than when given before adriamycin ($P<0.05$; Figure 2A).

In the AN group, dense mononuclear cell infiltrates were found in the renal interstitium, consisting of macrophages, CD8+ cells, and CD4+ T cells. In the pre-IL-2 group, there was a reduced interstitial monocytic infiltrate of the kidney with minimal macrophage, CD8+ cells, and CD4+ T cells. In the post-IL-2 group, although there was less infiltrate than with AN alone, there was greater infiltrate than in mice that received pretreatment with the complex (Figure 3).

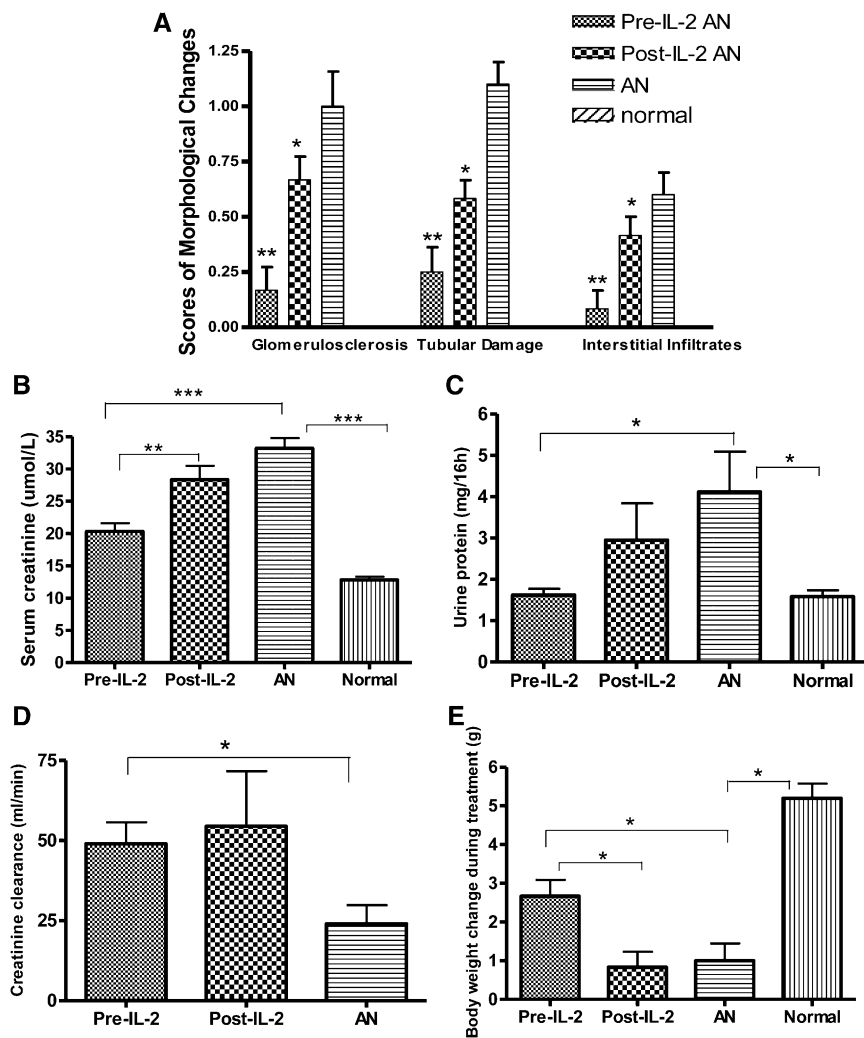


Figure 2. IL-2/IL-2Ab complex treatment protects against renal damage induced by adriamycin. Semiquantitative scores of morphologic changes of histopathology showed significantly less damage in glomeruli and tubules in pre-IL-2 group than in the AN group and the post-IL-2 group (A); mice from AN and post-IL-2 groups had significantly higher levels of serum creatinine (B) and urine protein excretion over 16 hours (C) and lower levels of creatinine clearance (D) compared with the normal (scores of morphologic changes for normal as designed as 0) and the pre-IL-2 groups. The pre-IL-2 group did not show the weight loss found in the other adriamycin-treated groups (E). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

To determine the mechanisms by which the IL-2/IL-2Ab complex protects against injury, we examined cytokine levels in spleens and kidney at day 35 after adriamycin administration. We observed a significant reduction in IL-6 and IL-17A mRNA levels in the pre-IL-2 group compared with the AN and post-IL-2 groups (Figure 4, C and D). *Foxp3* and IL-10, however, were higher in the pre-IL-2 group than in the AN and post-IL-2 groups (Figure 4, A and B). Thus, in

addition to higher levels of *Foxp3* consistent with Treg expansion, IL-2/IL-2Ab complex treatment led to reduction in inflammatory cytokines, including IL-6 and IL-17, and induction of protective anti-inflammatory cytokines, such as IL-10. This finding would be consistent with the IL-2/IL-2Ab group having direct expansion of Tregs (potentially the source of IL-10), which then limits ongoing inflammation, as reflected by improved histologic features and reduced

IL-6. The reduced IL-17 may then result from a lack of the cytokines required for Th17 differentiation, such as IL-6.

Furthermore, serum protein levels of IL-2, IL-4, IL-6, IFN- γ , TNF, IL-17A, and IL-10 were assessed. There was a significant increase in serum IL-2 at day 6 after IL-2 complex injection compared with saline treatment ($P < 0.001$; Figure 4E); however, in IL-2 complex-treated AN mice, IL-2 was at baseline at week 5 ($P < 0.05$; Figure 4F). In contrast, serum IL-6 at week 5 was increased in the AN group but was significantly lower in the pre-IL-2 and post-IL-2 groups ($P < 0.01$ and $P < 0.05$, respectively; Figure 4F). Serum IL-4, IFN- γ , TNF, IL-17A, and IL-10 were undetectable in all three groups of mice. This finding was similar to the pattern seen with RNA expression but was less sensitive than the RNA measurements; this may reflect activity within the kidney that was not present systemically. The cytokines IL-2 and TGF- β appear to be crucial for the development, expansion, and function of Tregs. Both the TGF- β SMAD signaling pathway through SMAD2 and SMAD3 and the IL-2 signal of transducer and activator of transcription-5 (STAT-5) signaling pathway have been shown to be important for *Foxp3* and Treg induction.¹⁰

IL-2 is known to activate the STAT pathway.^{16,17} Therefore, we evaluated the degree of STAT-5 activation by IL-2 or the IL-2/IL-2Ab complex through phosphorylation of STAT-5 in CD4 T cells. Murine splenocytes were treated with PBS, IL-2, or IL-2/IL-2Ab complex and analyzed for phosphorylation of STAT5 by flow cytometry. *In vitro* IL-2 and IL-2/IL-2Ab complex treatment produced a rapid increase in the phosphorylation of STAT-5. Of CD4+ T cells, $15\% \pm 3\%$ showed phosphorylation after 20 minutes of IL-2 treatment, whereas $30\% \pm 4\%$ showed phosphorylation of STAT-5 after 20 minutes with IL-2/IL-2Ab complex treatment (Figure 4G).

Tregs appear to be protective in AN, as shown by antibody removal using the anti-CD25 antibody PC-61 or by therapeutic injection of natural Tregs or *Foxp3*-transduced Tregs.^{4,5} In this model, although Treg expansion is only transient,

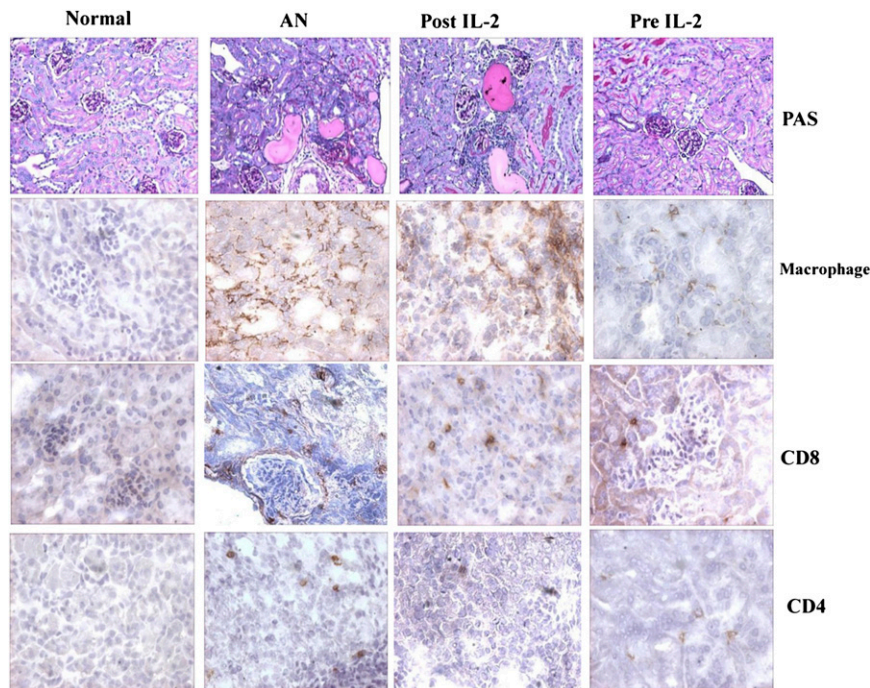


Figure 3. IL-2/IL-2Ab complex treatment reduces renal damage in AN. Representative histologic sections stained with periodic acid-Schiff (PAS) showed reduced renal injury and interstitial cellular infiltration in mice receiving IL-2/IL-2Ab complex injection (magnification $\times 200$). There was reduced interstitial monocyte infiltration with macrophage, CD8+, and CD4+ T cells in both pre-IL-2 and post-IL-2 groups compared with the AN group (original magnification $\times 400$).

the effect is maintained; this finding is in keeping with the results in EAE and islet transplantation.¹³ This finding also suggests that the expanded Tregs have a profound and long-lasting beneficial effect on inflammation in AN. The lesser effect found in the post-treatment group may be the result of expansion of CD25-expressing effector T cells in addition to Treg expansion, as previously described in the EAE model.¹³

The Treg-directed role of the IL-2/IL-2Ab complex may be functionally different from, and therapeutically superior to, the CD28-mediated expansion that was unsuccessful in human trials. Ongoing experiments in mouse models with a novel CD28 agonist suggest an increase in inflammatory activity with such compounds that necessitates steroid suppression; thus, their clinical use is highly unlikely because of associated inflammatory risks.¹⁷ The importance of this pathway clinically has been emphasized by the recent reports of low-dose IL-2 providing protection in human trials

against hepatitis C injury and graft-versus-host disease. The mechanism of this was due to expansion of Treg populations, suggesting that IL-2 treatment may be a promising approach for autoimmune renal disease in which Tregs play a protective role.^{18–20}

In conclusion, *in vivo* expansion of Tregs with IL-2/IL-2Ab complex leads to protection against chronic proteinuric renal injury and suggests that this may be a promising avenue for further therapeutic studies aimed at avoiding the general T cell activation found in human trials of Treg expansion with CD28 agonists.

CONCISE METHODS

Mice

Male BALB/c mice were obtained from the Animal Resources Centre in Perth, Australia. All mice were maintained free of pathogens in the Westmead Hospital Animal House. Eight-week-old mice (weight, 20–22 g) were used in all groups. Experiments were carried

out in accordance with protocols approved by the Animal Ethics Committee of Western Sydney Local Health District.

Induction of AN and IL-2/IL-2Ab Complex Administration

Male BALB/c mice were divided into four groups, each consisting of six mice: AN (adriamycin only), pre-IL-2 (adriamycin + IL-2/IL-2Ab complex administered before adriamycin), post-IL-2 (adriamycin + IL-2/IL-2Ab complex administered after adriamycin) and normal (control group, injected with saline). AN was induced by a single tail-vein injection of adriamycin (9.8 mg/kg, doxorubicin hydrochloride; Pharmacia & Upjohn, Perth, Australia). Pre-IL-2 mice were intraperitoneally injected with IL-2/IL-2Ab complex (1 μ g/5 μ g) daily for 3 days 1 week before adriamycin administration. Post-IL-2 mice were injected daily at days 5, 6, and 7 and days 12, 13, and 14 after adriamycin using the same doses as for pre-IL-2 mice.

Renal Function

Blood, spleen, and kidney samples were obtained from mice in each group on day 35. Renal function was assessed by 16-hour measurement of urine protein and serum creatinine using methods described elsewhere.⁴

RNA Isolation and Real-Time PCR and RT-PCR

RNA was isolated from mouse cells using TRIzol (Invitrogen, Life Technologies, Australia) according to the manufacturer's instructions. The total amount of RNA was reverse-transcribed using Superscript II reverse transcriptase (Invitrogen, Life Technologies) and random primers (Promega, Madison, WI) in a final volume of 20 μ l. PCR amplification was tested on each primer pair at different cycles to ensure amplification in the linear range.

Foxp3, IL-10, IL-6, and IL-17A mRNA levels were quantified by real-time PCR using TaqMan Gene Expression (Applied Biosystems) and Rotor-Gene software (Corbett Research). Glyceraldehyde 3-phosphate dehydrogenase was used as control. PCR condition and primers were as described elsewhere.⁴

Intracellular Staining for STAT5

Splenocytes were stained with anti-CD4 FITC and anti-CD25 allophycocyanin (BD

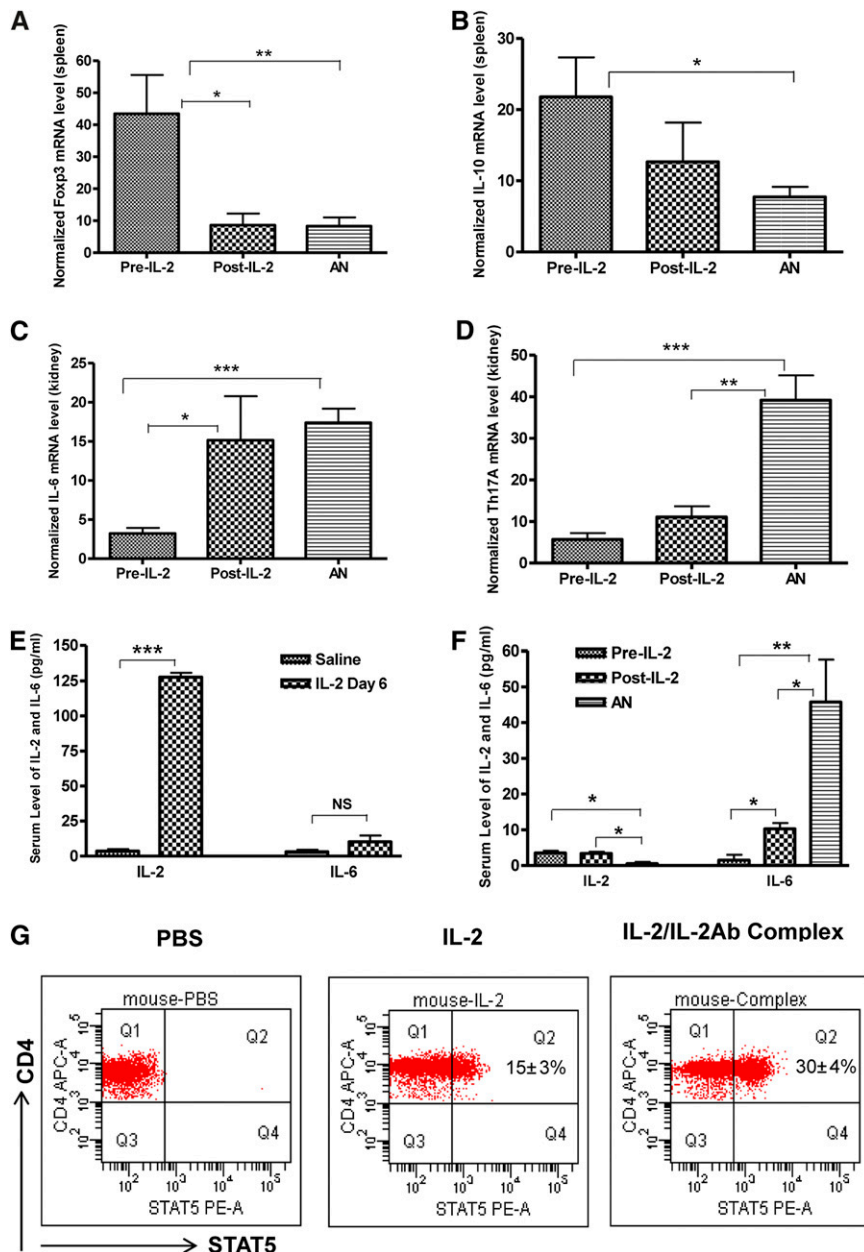


Figure 4. Real-time quantitative PCR of cytokine and *Foxp3* mRNA levels from spleen and kidney. At the completion of the AN model, the pre-IL-2 group had significantly higher mRNA levels of *Foxp3* (A) and IL-10 (B) in spleen and lower levels of IL-6 (C) and Th17A (D) than did the AN and post-IL-2 groups. Serum cytokine levels of IL-2 significantly increased at day 6 after IL-2 complex injection compared with saline-treated mice (E). However, at the completion of the AN, serum IL-2 returned to baseline. In contrast, IL-6 was elevated in the AN group but was significantly lower in both IL-2-treated groups (F). Murine splenocytes treated with IL-2 (100 ng/ml) and IL-2/IL-2Ab complex (IL-2, 100 ng/ml; anti-IL-2, 500 ng/ml of anti-IL-2; preincubated for 30 minutes at 37°C) *in vitro* showed a rapid increase in phosphorylation of STAT5. Phosphorylation was seen in 15%±3% of CD4+ T cells after 20 minutes of IL-2 stimulation; 30%±4% of CD4+ T cells showed phosphorylation of STAT5 after 20 minutes of IL-2/IL-2Ab complex treatment (G). Mean ± SD; **P*<0.05, ***P*<0.01, ****P*<0.001; *n*=5. PE-A, phycoerythrin.

Biosciences). The cells were treated with PBS, IL-2 (100 ng/ml), or IL-2/IL-2Ab complex (IL-2, 100 ng/ml; anti-IL-2, 0.5 µg/ml) for 20 minutes at 37°C.²¹ The cells were fixed with Lyse/Fix Buffer (BD Biosciences) for 10 minutes at 37°C, permeabilized by Phosflow Perm Buffer III (BD Biosciences) on ice for 30 minutes, and then stained with phycoerythrin mouse anti-phosphoSTAT5 (BD Biosciences).

BD Cytometric Bead Array

The BD CBA Mouse Th1/Th2/Th17 Cytokine Kit was used to detect IL-2, IL-4, IL-6, IFN-γ, TNF, IL-17A, and IL-10 serum protein, following the manufacturer's instructions (BD Bioscience). The data were analyzed by FACS Array software (BD).

Flow Cytometric Analysis

Antibodies used for flow cytometry included FITC-conjugated antimouse CD25 and allophycocyanin-conjugated antimouse CD4 (BD Bioscience). *Foxp3* intracellular staining was performed by using phycoerythrin anti-mouse/rat *Foxp3* Staining Set (eBioscience, clone: FJK-16s), following the manufacturer's instructions. All samples were analyzed on a LSRII-10 Color Flow Cytometer (Becton Dickinson, Mountain View, CA). BD FACS-Diva software was used for acquisition and analysis (Becton Dickinson, Australia).

Histology and Morphometric Evaluation

The kidneys were removed on day 35. Kidneys were fixed in neutral buffered formalin at room temperature for 24 hours, embedded in paraffin stained with periodic acid-Schiff reagent, and assessed by light microscopy. Cortex of the kidney was snap frozen in liquid nitrogen for RNA and immunohistochemistry using Optimal Cutting Temperature media (Sakura Finetek Inc., Torrance, CA). The degree of renal injury was assessed by two blinded observers as described elsewhere.⁴

Immunohistochemistry

Immunohistochemical staining was performed for *Foxp3*+ cells (eBioscience, antimouse *Foxp3*, clone FJK-16s). Secondary antibody used was biotinylated rabbit antirat immunoglobulin (Dako Corporation, Carpinteria, CA), and 5-µm sections were cut. Endogenous peroxidase activity and endogenous avidin binding activity were blocked. Normal rat

immunoglobulin was used for control. Sections were incubated with secondary antibodies and 3,3-diaminobenzidine (Dako). Slides were counterstained with hematoxylin (Sigma). For assessment of interstitial infiltration, positively stained cells located in interstitium only were counted from five random cortical fields (magnification $\times 400$) in each section, and the numbers were averaged for each field. Glomerular macrophage infiltration was evaluated as the number of macrophages per 10 glomerular cross-sections.

Statistical Analyses

Statistical analyses were performed by one-way ANOVA. Results are expressed as the group mean \pm SD. Two group differences were analyzed by *t* test. A *P* value (two-tailed) < 0.05 (GraphPad Prism software V4) was considered to represent a statistically significant difference.

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DISCLOSURES

None.

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