

A Superagonistic Monoclonal Antibody for CD28 Ameliorates Crescentic Glomerulonephritis in Wistar-Kyoto Rats

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Regulatory T (Treg) cells play an important role in the resolution of crescentic glomerulonephritis, where a T helper 1 (Th1)-predominant immune response promotes crescent formation. Therefore, agents that increase Treg cells appear to be ideal for suppressing T-cell-mediated renal pathology. We hypothesized that a superagonistic monoclonal antibody for CD28 (JJ316), which has been known to preferentially expand Treg cells *in vivo*, could prevent nephrotoxic serum-induced nephritis in Wistar-Kyoto rats, one of the experimental models of crescentic glomerulonephritis. Administration of JJ316 attenuated crescent formation, proteinuria and glomerular accumulation of macrophages and CD8⁺ T cells. These changes were accompanied by increased infiltration of Treg cells. Among glomerular macrophages, the CD163⁺ subset was significantly increased after treatment, suggesting that Treg cells may modulate the phenotype of macrophages leading to resolution of glomerulonephritis. In an adoptive transfer experiment, two T-cell subsets (CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells) purified from spleens and lymph nodes of donor rats primed with JJ316 3 d before were inoculated into nephritic recipient rats, which recapitulated the beneficial effects of *in vivo* administration of JJ316. Furthermore, a single injection of JJ316 administered 3 d after disease induction completely protected nephritic rats from death for 2 months. In conclusion, we demonstrated that treatment with JJ316 has a dramatic therapeutic effect on an experimental crescentic glomerulonephritis, possibly due to expansion and activation of Treg cells.

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INTRODUCTION

Naturally arising regulatory T (Treg) cells are a functionally distinct T-cell subpopulation that plays a pivotal role in the maintenance of self-tolerance in rodents and humans (1). They constitutively express CD25 (2) and more specifically express Foxp3, a key transcription factor, in their development and function (3). Treg cells are actively engaged in the negative control of a wide variety

of immune responses, including the prevention of autoimmune diseases (4,5), the induction of immunological tolerance to non-self-antigens (such as transplantation tolerance [6]) and negative control of aberrant immune responses (such as allergy [7]). Accumulating evidence supporting the role of Treg cells in the maintenance of immune homeostasis has prompted researchers to investigate their therapeutic potential. Al-

though progress has been made in expanding Treg cells *in vitro* and infusing them into diseased animals (8–10), *in vivo* induction of Treg activity may be more practical and valuable for use in a clinical setting.

Under natural conditions, T-cell activation requires two separate stimulatory signals from antigen-presenting cells. The first signal occurs via a T-cell receptor and a costimulatory signal via CD28. To mimic physiological T-cell activation *in vitro*, monoclonal antibodies (mAbs) for CD28 were developed. However, “conventional” anti-CD28 mAbs require first signals via the T-cell receptor to fully activate T cells, whereas the mAb for rat CD28, called JJ316 (mouse IgG1, κ), is capable of fully activating T cells

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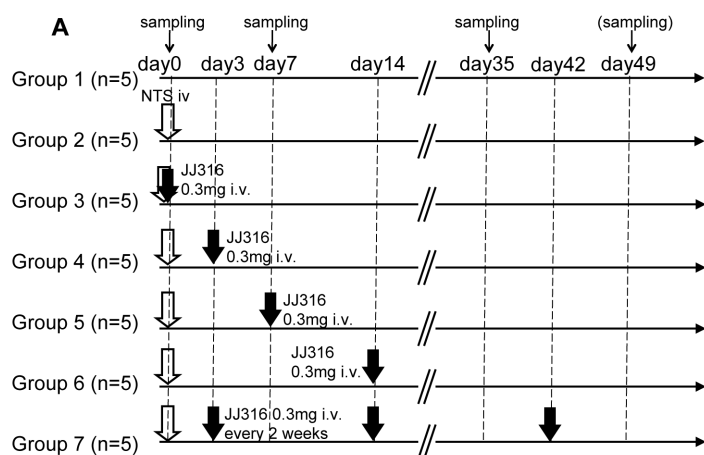


Figure 1. Experimental protocol of the long-term experiment. i.v., intravenous.

without additional stimulation, which makes this mAb a “superagonist” (11–14).

Crystallographic analyses of a homologous anti-human CD28 superagonist reveal that these mAbs bind CD28 bivalently, which may lead to the stable lattice formation on the T-cell membrane, allowing aggregation of intra-signaling components and thus the production of strong activating signals (14). Most notably, *in vivo* administration of this superagonist induces preferential expansion of Treg cells (15,16), which leads to the attenuation of several experimental autoimmune diseases, including experimental autoimmune neuritis (17) and experimental autoimmune encephalomyelitis (16).

A pivotal role for adaptive immune response in the initiation and development of glomerulonephritis was demonstrated in many experimental models. The strongest case that adaptive immune responses drive nephritogenic events in glomeruli is the crescentic glomerulonephritis model, where local CD4⁺ T-cell-driven Th1-type responses play a predominant role in both the initiation and the effector phase of the disease (18). On the basis of this background, we hypothesized that a superagonistic mAb specific for rat CD28 (CD28-SA), JJ316, would prevent experimental crescentic nephritis, and we examined the therapeutic effect of JJ316 using a rat model

initiated by heterologous anti-glomerular basement membrane (GBM) globulin (nephrotoxic serum [NTS]-induced nephritis).

MATERIALS AND METHODS

Animals and the Nephritis Model

Seven-week-old WKY (Wistar-Kyoto)/NCrj inbred rats, weighing approximately 150–200 g, were purchased from Charles River Laboratories Japan (Osaka, Japan). Rabbit-derived NTS used in this experiment induced aggressive crescentic nephritis without the need for preimmunization, and nephritic rats died owing to uremia in up to 2 months (19). In all animal experiments, rats were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg) and handled in a humane fashion in accordance with the guidelines of the Animal Committee of Osaka University.

Experimental Protocols

Effect of the superagonist on nephritis in a short-term experiment. For protocol 1-1, NTS was injected on day 0 into WKY rats, and on days 3, 0.1, 0.3 or 1 mg JJ316 or the same isotype mAb (MOPC-31c) was injected into the rats ($n = 10\text{--}12$ in each group). Rats were harvested on day 8 ($n = 6\text{--}7$ in each group) to examine renal pathology, urinary protein level, infiltration of Treg cells, CD8⁺ T cells and macrophages, and immunoglobulin dep-

osition in the glomeruli and on day 5 ($n = 4\text{--}6$ in each group) to examine glomerular mRNA expressions of pro-inflammatory cytokines.

For protocol 1-2, rats were injected with NTS or normal rabbit serum, and half of the rats in both groups were administered 1 mg JJ316 at the same time ($n = 4$ in each group). Three days after induction of nephritis, draining lymph nodes (LNs) were removed and mRNA levels of cytokines and transcription factors were estimated by real-time polymerase chain reaction (PCR).

Adoptive transfer experiment. For protocol 2, healthy donor rats were primed with 1 mg JJ316, and 3 d later, single-cell suspensions were obtained from their spleens and LNs. Thereafter, two T-cell subsets (CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells) were purified using MACS separation columns. Recipient rats were inoculated with saline, CD4⁺CD25⁺ T cells (0.2×10^7 or 2×10^7) or CD4⁺CD25⁻ T cells (2×10^7) from donor rats ($n = 4\text{--}5$ in each group). Rats were injected with NTS on the same day and sacrificed 8 d later to investigate renal pathology and urinary protein level.

Effect of the superagonist on nephritis in a long-term experiment. For protocol 3, WKY rats were divided into seven groups ($n = 5$ in each group) (Figure 1). Rats in groups 1 and 2 were allocated to healthy and disease controls, respectively. After injection of NTS on day 0, 0.3 mg JJ316 was injected into WKY rats on day 0 (group 3), day 3 (group 4), day 7 (group 5) or day 14 (group 6) and every 2 wks from day 3 (group 7). We collected blood and urine from all rats on days 0, 7, 21, 35 and 49 and evaluated their body weight, renal function, urinary protein and survival rates.

Reagents and Antibodies Used in FACS or Immunohistochemical Analysis

The following mAbs and reagents were used: allophycocyanin (APC)-conjugated antirat CD4 (OX35; BD Biosciences, San Diego, CA, USA); fluores-

cein isothiocyanate (FITC)-conjugated antirat CD25 (OX39; BD Biosciences); phycoerythrin (PE)-or biotin-conjugated anti-FoxP3 (FJK-16s; eBioscience, San Diego, CA, USA); unconjugated or Alexa488-conjugated antirat CD68 (ED1; AbD Serotec, Oxford, U.K.); biotin-conjugated antirat CD163 (ED2; AbD Serotec); antirat CD8a (OX-8; BD Biosciences); Alexa488-conjugated antirabbit IgG (Molecular Probes, Carlsbad, CA, USA); Alexa555-conjugated antirat IgG (Molecular Probes); biotin-conjugated antirat IgG1/2a (BD Biosciences); FITC-conjugated antirat IgG2b (BD Biosciences); Alexa555-conjugated streptavidin (Molecular Probes); and mouse IgG1, κ (MOPC-31c; Sigma, St. Louis, MO, USA).

FACS Analysis

Single-cell suspensions of mononuclear cells were prepared from spleens and LNs of WKY rats using Lymphoprep (Nycomed, Oslo, Norway). Approximately 10^6 cells resuspended in 1 mL phosphate-buffered saline/2% fetal bovine serum were incubated with APC-conjugated anti-CD4 antibody (1:200) and FITC-conjugated anti-CD25 antibody (1:200) (30 min, 4°C). In some experiments, the cells were fixed for 60 min with fixation/permeabilization buffer (eBioscience) and then incubated for 60 min with permeabilization buffer (eBioscience). PE-conjugated anti-FoxP3 antibody was added at the concentration of 1 $\mu\text{g}/\text{mL}$ and incubated for 30 min at 4°C. The cells were analyzed after a final washing step on a FACS Calibur flow cytometer by using Cell Quest software (all BD Biosciences).

Purification of CD4⁺CD25⁺ and CD4⁺CD25⁻ T Cells

Single-cell suspensions were prepared from spleens, and inguinal, axillary and para-aortic LNs. Two T-cell subsets (CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells) were purified using MACS (Miltenyi Biotec, Bergisch-Gladbach, Germany) separation columns according to the manufacturer's instructions. In brief,

single-cell suspensions were stained with APC-conjugated anti-CD4 antibody and FITC-conjugated anti-CD25 antibody. Subsequently, CD4⁺ cells were positively selected after labeled with anti-APC MultiSort MicroBeads (Miltenyi Biotec). CD4⁺CD25⁺ cells were positively selected after being magnetically labeled with anti-FITC MicroBeads. Cell purities of regulatory CD25⁺ T cells and conventional CD25⁻ T cells were, on average, 88% and 92%, respectively.

Morphological Studies

Kidneys were fixed in 4% periodate-lysine-paraformaldehyde and were paraffin embedded. Two- μm sections were stained with the periodic acid-Schiff (PAS) reagent. Crescent formation in the nephritic glomeruli was evaluated as a proportion of the numbers of crescentic glomeruli relative to those of total glomeruli.

Immunohistochemical Studies

For immunohistochemical staining for FoxP3, CD8 and CD68, kidneys or draining LNs from kidneys were fixed in 4% periodate-lysine-paraformaldehyde for ~18 h and were paraffin embedded. Immunohistochemical staining was performed using a streptavidin biotin-staining method (Vector ABC Kit; Vector Laboratories, Burlingame, CA, USA). For both stainings, sections were autoclaved in 0.01 mol/L citrate buffer (pH 6.0) for 10 min at 121°C to retrieve the antigen. Infiltration of the cells positive for these markers into the nephritic glomeruli was calculated as a proportion of the numbers of positively stained cells relative to those of total glomeruli.

For costaining of CD68 (ED1)⁺ and CD163 (ED2)⁺ cells, kidney specimens were fixed with zinc fixative (BD Biosciences) for ~18 h and were paraffin embedded. Sections were first stained for CD163 using biotin-conjugated antibody and Alexa555-conjugated streptavidin. Thereafter, CD68 was stained using an Alexa488-conjugated anti-CD68 antibody. Slides were analyzed using confocal mi-

croscopy (Radiance 2100; Carl Zeiss, Jena, Germany).

For immunohistochemical staining for rat and rabbit IgG, kidney sections were embedded in OCT compound and snap frozen in liquid nitrogen. Four- μm sections were stained with Alexa555-conjugated antirat IgG, FITC-conjugated antirat IgG1 + 2a (which recognizes both rat IgG1 and IgG2a) or FITC-conjugated antirat IgG2b without fixation.

Measurement of Urinary Protein Excretion Level and Renal Function

Spot urine was collected from control and treated rats and urinary protein, and creatinine level was measured. Therapeutic efficacy was evaluated as a ratio of protein concentration relative to creatinine concentration. Renal function was evaluated by the serum creatinine and urea nitrogen level. The Micro TP-AR (Wako Pure Chemical Industries, Osaka, Japan) was used to measure total protein by pyrogallol red method on the automatic analyzer (Hitachi). Serum and urinary creatinine were determined by enzymatic creatinine assay using NescotTM VLII CRE reagent (Alfresa Pharma Corporation, Tokyo, Japan). Serum urea nitrogen level was measured by using the urease-GLDH method and the UN-S reagent (Denka Seiken, Tokyo, Japan).

RNA Extraction and Real-Time PCR

Total RNA was extracted from isolated glomeruli, draining LNs and spleens using the Trizol reagent according to the manufacturer's instruction (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized from 0.4 μg total RNA using the SuperScript First Strand Synthesis System for RT-PCR (Invitrogen). Relative quantitation of the expression levels of various molecules was carried out with PCR using a set of oligonucleotide primers (Table 1).

Statistical Analyses

All values are expressed as means \pm SD. Statistical significance was evaluated

Table 1. Primer sequences used for real-time PCR analysis.

Gene	Forward	Reverse
IL-6	5'-CCGGAGAGGAGACTTCACAG-3'	5'-ACAGTGCATCATCGCTGTTC-3'
TNF α	5'-AGTCCGGGCAGGTCTACTTT-3'	5'-CGTGTGTTTCTGAGCATCGT-3'
MCP-1	5'-ATGCAGTTAATGCCCCACTC-3'	5'-TTCCTTATTGGGGTCAGCAC-3'
IFN γ	5'-GCCCTCTCTGGCTGTACTG-3'	5'-CTGATGGCCTGGTGTCTTT-3'
IL-4	5'-TCCTTACGGCAACAAGGAAC-3'	5'-GTGAGTTCAGACCGCTGACA-3'
IL-10	5'-GGGAAGCAACTGAACTTCG-3'	5'-GCTTTCGAGACTGGAAGTGG-3'
T-bet	5'-CCTGGACCCAACCTGTCAACT-3'	5'-CGGTAGTAGGGCACAGTGGT-3'
GATA3	5'-GAGAGCAGGGACTTCTGTG-3'	5'-CATCATGCACCTTTTGCAC-3'
FoxP3	5'-CGGGAGAGTTTCTCAAGCAC-3'	5'-GGAGCTCTGTCCACTGAGG-3'
β -Actin	5'-TGTACCAACTGGGACGATA-3'	5'-GGGGTGTGAAGGTCTCAAA-3'

using the nonpaired *t* test. A *P* value of <0.05 was considered significant.

RESULTS

Preferential Expansion of Treg Cells by *In Vivo* Administration of the Superagonist

To investigate the effect of JJ316 on the bias of Treg cells and non-Treg cells, normal WKY rats were injected with three different amounts of JJ316 (0.1, 0.3 and 1 mg per animal) (*n* = 4–5 in each group). Rats in the control group were injected with the same IgG isotype (MOPC-31c). We have chosen the doses of JJ316 on the basis of our former experiments (15,16). Three days after administration, a single-cell suspension from the total splenocytes and LN cells was analyzed by flow cytometry after staining for CD4 and CD25. The proportion of CD4⁺CD25⁺ T cells relative to overall CD4⁺ T cells in splenocytes is demonstrated in Figure 2A (*upper*) and in Figure 2B; for the LN cells, see Figure 1A (*lower*) and Figure 2C. The absolute numbers of CD4⁺CD25⁺ T cells in spleen after administration of JJ316 is shown in Figure 2D.

The fraction of CD4⁺CD25⁺ cells increased up to 2.5 (1.7)-fold, 3.3 (2.5)-fold and 3.5 (2.2)-fold in spleen (LN) with administration of 0.1, 0.3 and 1 mg JJ316, respectively. The absolute numbers of CD4⁺CD25⁺ cells increased up to 2.4-, 16.3- and 21.4-fold in spleen with administration of 0.1, 0.3 and 1 mg JJ316, respectively, whereas those of CD4⁺CD25⁻ cells increased up to 0.9-, 4.1- and 5.0-fold. Administration of higher amounts

of JJ316 (0.3 or 1 mg) induced more pronounced expansion of CD4⁺CD25⁺ T cells, but was accompanied by less co-expansion with other CD4⁺ T-cell subpopulations (Figure 2D, E). Lower amounts of JJ316 (0.1 mg) selectively expanded CD4⁺CD25⁺ T cells without affecting the CD4⁺CD25⁻ T-cell fraction, although the number of CD4⁺CD25⁺ T cells was smaller compared with the high-dose group.

Because the non-Treg cells, in principle, can express CD25 after stimulation, we explored the expression of FoxP3 and found that ~82–94% of CD4⁺CD25⁺ cells express FoxP3, which confirmed our hypothesis that most of the expanded CD4⁺CD25⁺ T cells are Treg cells (Figure 2F). Furthermore, we investigated the expansion of FoxP3⁺ Treg cells in spleen (data not shown) and LN (Figure 2G) by immunohistochemistry, which confirmed the FACS data. These data are in good agreement with results previously reported for Lewis (LEW) rats (16) and suggest that *in vivo* administration of JJ316 leads to the expansion of Treg cells preferentially at high doses and more selectively at low doses.

Effect of the Superagonist on Nephritis in a Short-Term Experiment

On the basis of these results, we examined the therapeutic effect of JJ316 using a rat NTS-induced nephritis model, in which effector T cells play a predominant role. We performed a short-term experiment according to the experimental protocol (protocol 1-1).

First, we assessed the renal pathology and urinary protein levels. Eight days

after the induction of nephritis, crescents were seen in about 60% of glomeruli in rats in the disease group, whereas <10% of glomeruli were crescentic in rats injected with 0.3 and 1 mg JJ316 (Figure 3A, B). Although 0.1 mg JJ316 was also effective, the effect was weak when compared with the high-dose group (see Figure 3B). Urinary protein excretion was also significantly diminished in treated rats and the dose-dependency of JJ316 was similar to that seen with crescent formation (Figure 3C).

Figure 4A (*upper*) shows the immunohistochemical staining for FoxP3 in the nephritic kidney on day 8, with or without the administration of 1 mg JJ316. In the untreated kidney, few Treg cells were seen in the glomeruli, whereas numerous Treg cells infiltrated the treated kidney. Statistical analysis demonstrated that the number of infiltrated Treg cells were significantly increased with administration of JJ316 at all doses compared with the untreated kidney and that higher amounts of JJ316 induced more pronounced infiltration when compared with low doses (Figure 4B, *upper*). Immunohistochemical staining for CD8 (expressed on cytotoxic T cells) and CD68 (ED1; expressed on macrophages) was performed on JJ316-treated and untreated kidney specimens (see Figure 4A [*middle and lower*]). Statistical analysis (see Figure 4B [*middle and lower*]), as assessed by the numbers of cells per glomeruli, demonstrated that there was a significant decrease in the infiltration of cytotoxic T cells and macrophages in all JJ316-treated groups, although substantial infiltration by macrophages was seen, even after treatment.

We next investigated the expression of CD163 (ED2), which has been reported to be expressed in alternatively activated macrophages, by using immunohistochemistry (Figure 4C). We found that the ratio of the number of CD163⁺ cells among that of CD68⁺ cells significantly increased after administration of JJ316.

To explore the expression of pro-inflammatory cytokines, which are potential contributors during effector

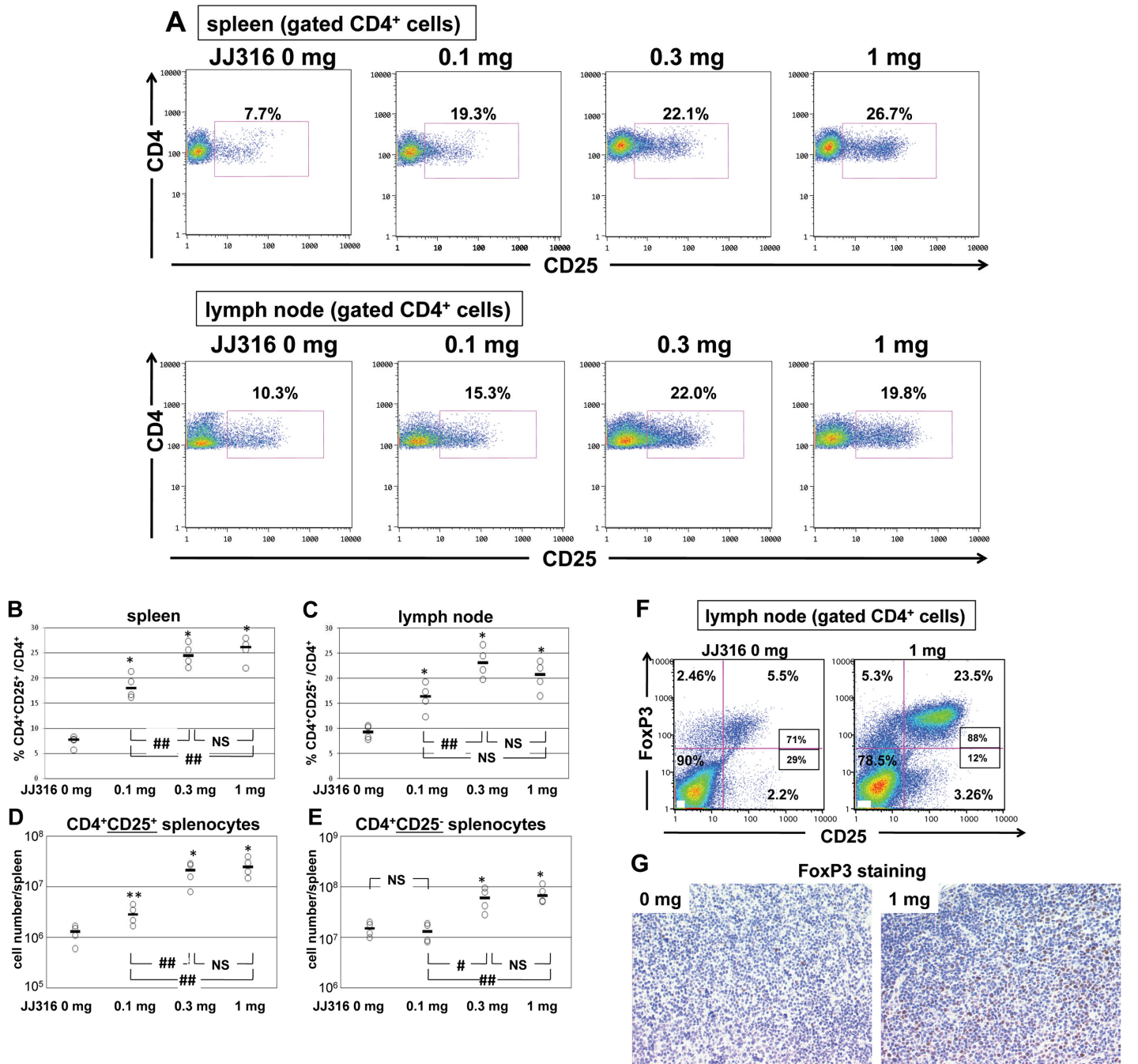


Figure 2. Preferential expansion of Treg cells by *in vivo* administration of JJ316. Splenocytes and LN cells of normal WKY rats were examined 3 d after administration with three different amounts of JJ316 (0.1, 0.3 or 1 mg per animal) or with the same isotype control mAb (MOPC-31c) (n = 4–5 in each group). (A–F) Single-cell suspensions were prepared from total splenocytes and LN cells and analyzed by flow cytometry after staining for CD4, CD25 and FoxP3. (A) Representative dot plots of CD4 and CD25 expression in spleens (*upper*) and LNs (*lower*) of control or JJ316 (1 mg)-treated animals. The percentages of CD25⁺ cells among CD4⁺ cells are shown. (B, C) The proportion of CD25⁺ cells among CD4⁺ cells is summarized for all animals analyzed. (D, E) Absolute numbers of CD4⁺CD25⁺ (D) or CD4⁺CD25⁻ (E) splenocytes per animal were obtained by multiplying total mononuclear cell numbers with the percentages of respective cells obtained by FACS analysis. (B–E) Each circle represents one animal. Horizontal bars indicate medians. **P* < 0.01 and ***P* < 0.05 versus control (0 mg). #*P* < 0.01 and ##*P* < 0.05 between indicated groups. (F) Representative dot plots of CD25 and FoxP3 expression among CD4⁺ LN cells of control or JJ316 (1 mg)-treated animals. The percentages of cells in the respective quadrant are shown. (G) LNs of control (*left*) or JJ316 (1 mg)-treated (*right*) animals were immunostained for FoxP3. Original magnification 400x.

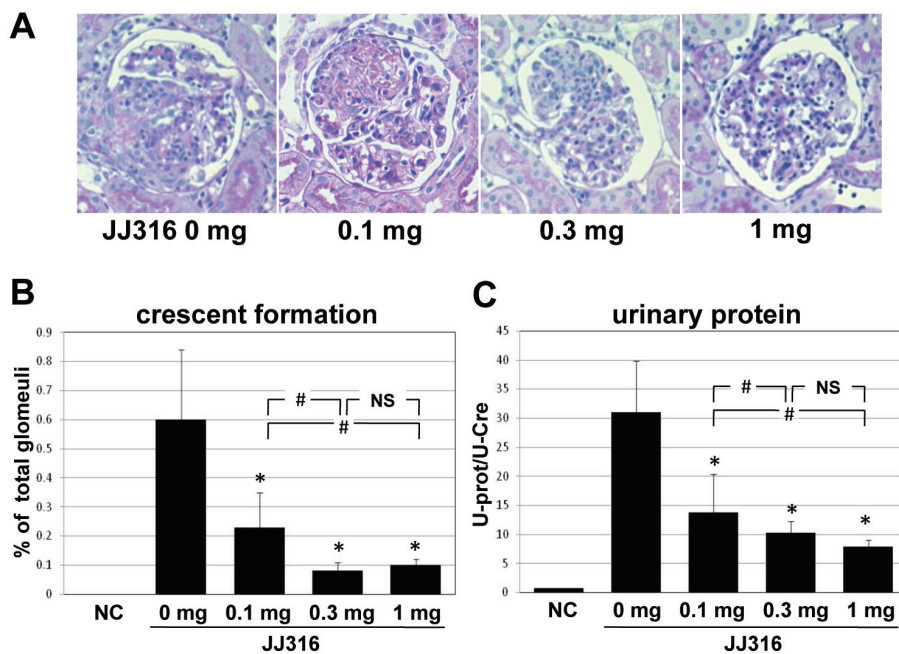


Figure 3. Effect of JJ316 on the renal pathology and urinary protein level in the NTS-induced nephritis model. The experiment was performed according to experimental protocol 1-1. (A) Representative images of PAS-stained glomerular sections of untreated and treated rats by JJ316 at the indicated doses. Original magnification 1,000 \times . (B) Ratios of crescent formation per 30 glomeruli of each rat (~200 glomeruli in each group) were investigated. Data were presented as means \pm SD. * P < 0.05 versus disease control (0 mg). # P < 0.05 between indicated groups. (C) Urinary protein level was assessed as the ratio of urinary protein to urinary creatinine concentration. Data were presented as means \pm SD. * P < 0.05 versus disease control (0 mg). # P < 0.05 between indicated groups. NC, normal healthy control.

T-cell-directed injury, glomerular mRNA expression of interleukin (IL)-6, tumor necrosis factor (TNF)- α and monocyte chemoattractant protein-1 (MCP1) on day 5 were investigated using real-time PCR (Figure 4D). We found that there was a significant reduction in the mRNA levels of all these proinflammatory cytokines after treatment with JJ316 (0.3 and 1 mg) compared with the disease group.

Because Treg cells are known to influence humoral immune responses, we evaluated the deposition of autologous and heterologous IgG on the GBM (Figure 5). Eight days after disease induction, deposition of rat IgG in treated groups was more prominent when compared with the disease group and exhibited a fine linear pattern along the GBM, whereas the deposition of rat IgG in the untreated kidneys was sparse and granu-

lar (Figure 5A). Furthermore, we explored the deposition of rat IgG1 and IgG2a isotypes using an antibody recognizing both IgG subclasses (Figure 5D) and that of rat IgG2b (Figure 5E). The results clearly show that the depositions of these IgG isotypes are also stronger in treated groups than in the disease group.

Finally, to investigate the effect of JJ316 on the bias between Th1, Th2 and Treg cells, we performed an additional series of short-term experiments according to the experimental protocol (protocol 1-2) and assessed mRNA levels of cytokines and key transcription factors by real-time PCR. We found that interferon (IFN)- γ (Th1 cytokine) was downregulated in LNs of both normal and nephritic rats after administration of JJ316, whereas IL-4 (Th2 cytokine) and IL-10 (Th2 and Treg cytokines) showed the opposite effect (Figure 6A). Furthermore, we found

that T-bet (key transcription factors for Th1) was downregulated in the LNs of normal and nephritic rats after treatment, whereas GATA3 and FoxP3 (key transcription factors for Th2 and Treg) were upregulated (Figure 6B).

Adoptive Transfer Experiment

To confirm that the therapeutic effect seen in the short-term experiment was truly mediated by superagonist-induced expansion of Treg cells, we next performed an adoptive transfer experiment according to the experimental protocol (protocol 2). Adoptive transfer of JJ316-induced Treg cells (2×10^7) clearly protected recipient rats, as assessed by renal pathology (Figure 7A, B) and urinary protein excretion (Figure 7C) when compared with the saline-inoculated rats. In contrast, CD4⁺CD25⁻ non-Treg cells or a small amount of CD4⁺CD25⁺ T cells (0.2×10^7) did not affect the pathology of nephritis.

Effect of the Superagonist on Nephritis in a Long-Term Experiment

We then performed a long-term experiment to study the effect of the superagonist on nephritis according to the experimental protocol (protocol 3) (see Figure 1). The renal function data as assessed by serum urea nitrogen and creatinine of rats in groups 1 (normal control), 2 (disease control), 4 and 7 demonstrated that untreated rats (group 2) became uremic, and urea nitrogen levels reached 100 mg/dL after 35 d, whereas renal function in rats in groups 4 and 7 was significantly preserved up to 49 d (Figure 8A, B), and body weight loss due to uremia was prevented in these groups (Figure 8C). Urinary protein level was also significantly diminished (Figure 8D). Survival rates demonstrated that injection of JJ316 once on day 3 or every 2 wks protected nephritic rats from death for >2 months (Figure 8E). Another analysis that focused on the timing of JJ316 injection (groups 2-6) clearly demonstrated that JJ316 treatment protected all nephritic rats from death when injected on day 0 or 3 and

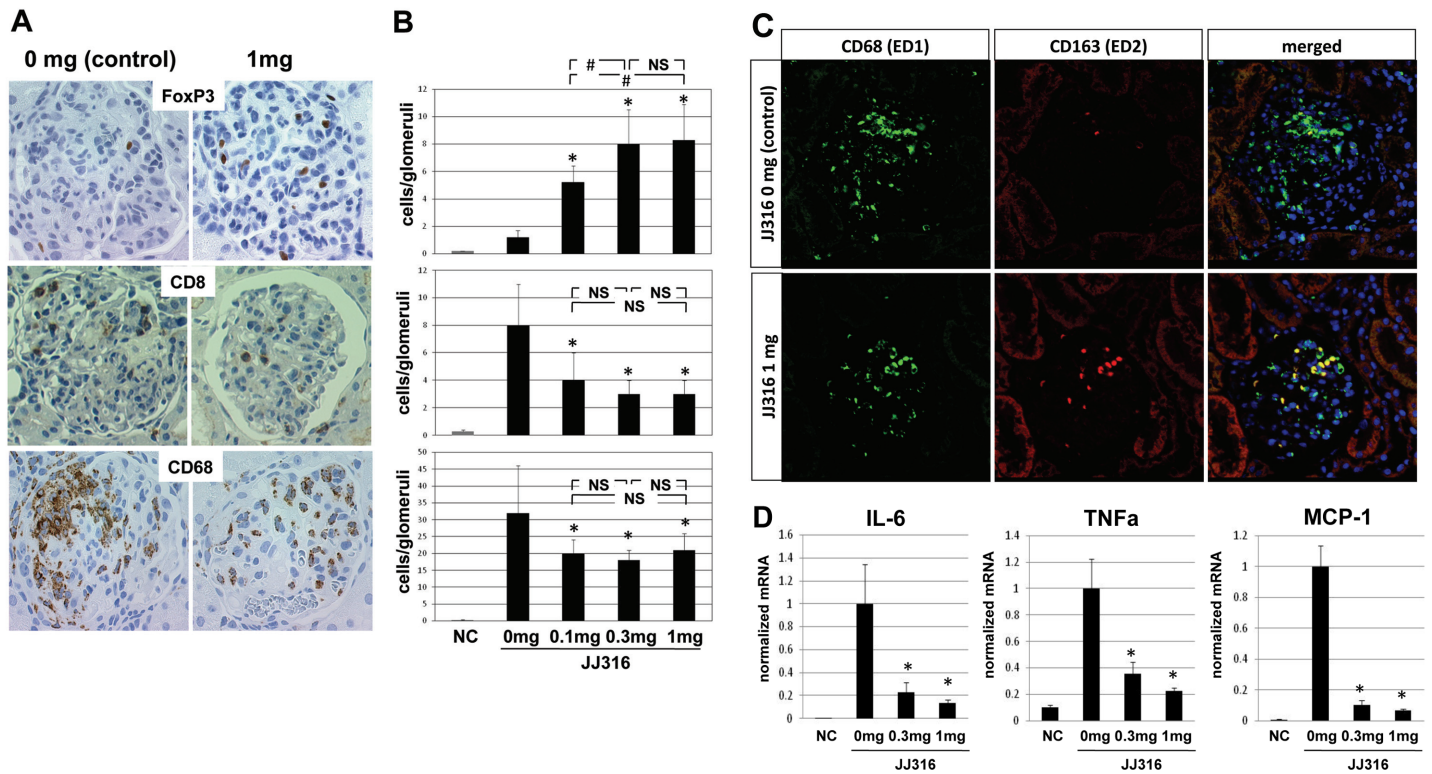


Figure 4. Effect of JJ316 on mononuclear cell infiltration and cytokine expression in the NTS-induced nephritis model. The experiment was performed according to experimental protocol 1-1. (A–C) Effect of JJ316 on infiltration into nephritic glomeruli by Treg cells, CD8⁺ T cells and macrophages. (A, B) Sections of periodate-lysine-paraformaldehyde-fixed and paraffin-embedded kidneys of rats (untreated and treated) were stained for FoxP3 (upper), CD8 (middle) and CD68 (ED1) (lower) and visualized using 3,3'-diaminobenzidine (DAB). (A) Representative glomerular images of untreated (left) and JJ316 (1 mg)-treated rats (right). Original magnification 1,000 \times . (B) Numbers of FoxP3⁺ Treg cells, CD8⁺ T cells and CD68⁺ cells infiltrating the nephritic glomeruli were counted using ~30–40 glomeruli in each group. Data were presented as means \pm SD. * P < 0.05 versus disease control (0 mg). # P < 0.05 between indicated groups. (C) Sections of zinc-fixed and paraffin-embedded kidneys of rats (untreated and treated) were costained for CD68 (ED1) and CD163 (ED2). Representative glomerular images of untreated (upper) and JJ316 (1 mg)-treated (lower) rats are shown. CD68⁺ and CD163⁺ cells are visualized in green (left) and red (middle), respectively, and merged images are shown in the right panels. Original magnification 1,000 \times . (D) Effect of JJ316 on proinflammatory cytokine expression in the glomeruli. Glomerular expression of IL-6, TNF α and MCP-1 was assessed by real-time PCR. Expression levels were normalized with β -actin mRNA and then normalized to disease control (0 mg). Data were presented as means \pm SD. * P < 0.05 versus disease control (0 mg).

provided partial protection when injected on day 7 (Figure 8F). When injected on day 14, therapeutic effects were not observed.

DISCUSSION

In this report, we demonstrate that the superagonistic monoclonal antibody for CD28 (CD28-SA), JJ316, preferentially expanded Treg cells in WKY rats and that administration of JJ316 to nephritic rats was effective in improving renal pathology, reducing proteinuria and, most importantly, delaying or preventing death.

Several reports have demonstrated the beneficial effects of cell therapy using Treg cells for renal disease. Injection of CD4⁺CD25⁺ T cells isolated from normal mice into severe combined immunodeficiency (SCID) mice with adriamycin nephropathy diminished renal damage (20). Adoptive transfer of Foxp3-transduced T cells protected against murine chronic renal injury from adriamycin (21). Transfer of isolated CD4⁺CD25⁺ T cells into mice that were subsequently injected with NTS significantly attenuated the development of nephropathy (22). The main issue is to

obtain sufficient numbers of Treg cells with potent suppressive activity against effector T-cell responses. Although several reagents have been shown to boost the pool or enhance the activity of Treg cells, the effect is limited or nonspecific. In light of this situation, application of JJ316, which increases the number of Treg cells between 2.4- to 21.4-fold in the spleen (in this study) and furthermore enhances the suppressor function of Treg cells at the single-cell level 10-fold (16), may provide a therapeutic strategy for the treatment of effector T-cell-induced renal disease. Therefore, we investigated

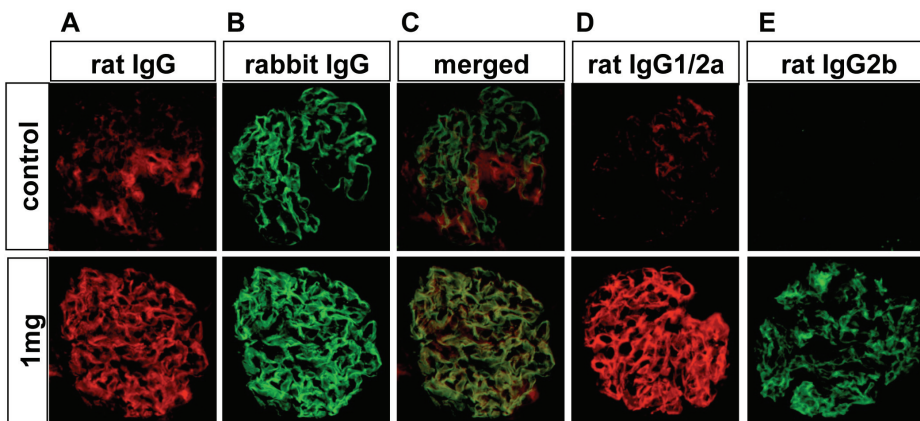


Figure 5. Effect of JJ316 on autologous and heterologous IgG deposition on the nephritic glomeruli of rats untreated (0 mg) and treated with 1 mg JJ316. The experiment was performed according to experimental protocol 1-1. Snap-frozen kidneys were sectioned and costained for rat IgG (A) in red and rabbit IgG (B) in green without fixation. Merged images are also presented (C). Other kidney sections were stained using antibodies that recognized rat IgG1 and IgG2a isotypes (D) in red or IgG2b isotype (E) in green. Original magnification 1,000 \times (A–E).

the effect of CD28-SA on NTS-induced nephritis, an experimental model of crescentic glomerulonephritis, in which Th1 predominant immune responses have been shown to promote crescent formation (18).

In the short-term experiment, JJ316 exhibited remarkable therapeutic effects on NTS-induced nephritic rats, as assessed by the improvement of renal pathology and the reduction of proteinuria. The therapeutic efficacy depended on the doses of JJ316. High doses of JJ316 of 0.3 or 1 mg were superior to low doses (0.1 mg), reflecting that the larger amounts of Treg cells overwhelmed the adverse effect that was due to coexpanded non-Treg cells. Although the therapeutic efficacy of JJ316 in this model could, in principle, be independent of the observed expansion of Treg cells, adoptive transfer experiments clearly revealed that it was at least partially mediated by superagonist-induced activation of Treg cells. It is an important question as to whether Treg cells generated in animals with or without glomerulonephritis are more efficacious in the adoptive transfer experiments. There was no difference in the therapeutic effect between these two Treg cell subsets (data not shown). A similar result was obtained in the rat encephalomyelitis model (16). Although this suggests that the preexisting natural T-cell receptor repertoire of Treg cells contains a sufficient frequency of cells that is reactive to the GBM–anti-GBM complex, it remains to be solved whether the therapeutic effect that was observed *in vivo* is mediated largely by Treg cells with specificity for the GBM–anti-GBM complex or achieved mainly by way of bystander suppression by Treg cells recognizing other antigens.

We observed that the depositions of autologous (rat) IgG and several IgG subclasses on the GBM are more prominent in treated groups compared with those of the disease group. This does not simply reflect the destruction of GBM because rabbit IgG shows largely intact GBM (Figure 5B). We have already reported that administration of CD28-SA

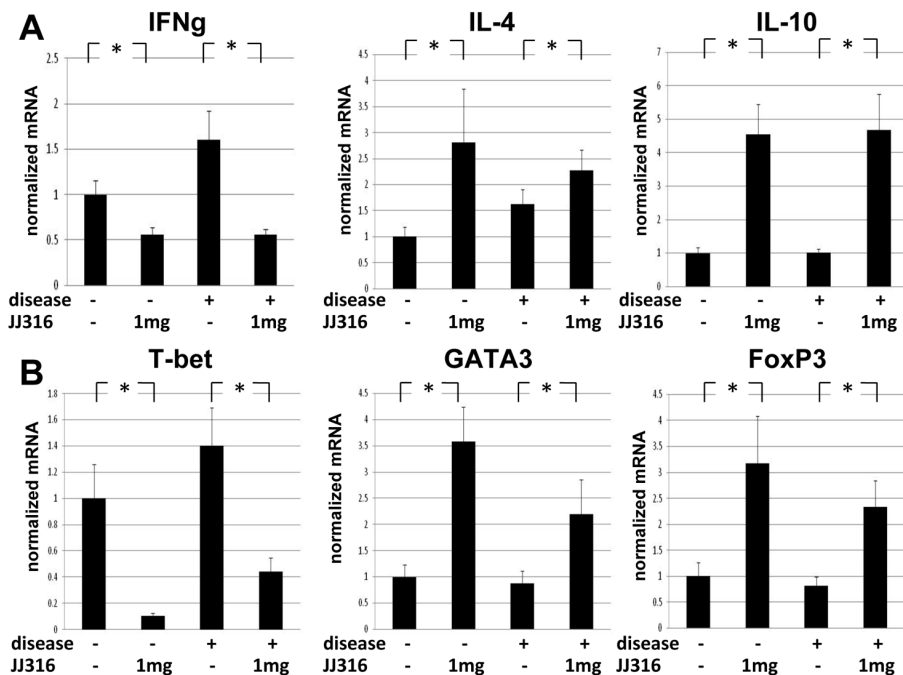


Figure 6. Effect of JJ316 on mRNA expression of cytokines and transcription factors in LN. The experiment was performed according to experimental protocol 1-2. (A) mRNA expression of IFN γ , IL-4 and IL-10, which are predominantly secreted by Th1, Th2 and Treg (and Th2) cells, respectively, was determined as the ratio relative to β -actin mRNA expression. (B) mRNA expression of T-bet, GATA3 and FoxP3 (which are key transcription factors of Th1, Th2 and Treg cells, respectively) was also determined as the ratio relative to β -actin mRNA expression. (A, B) Data were normalized to normal nondisease control and presented as means \pm SD. * P < 0.05 between indicated groups.

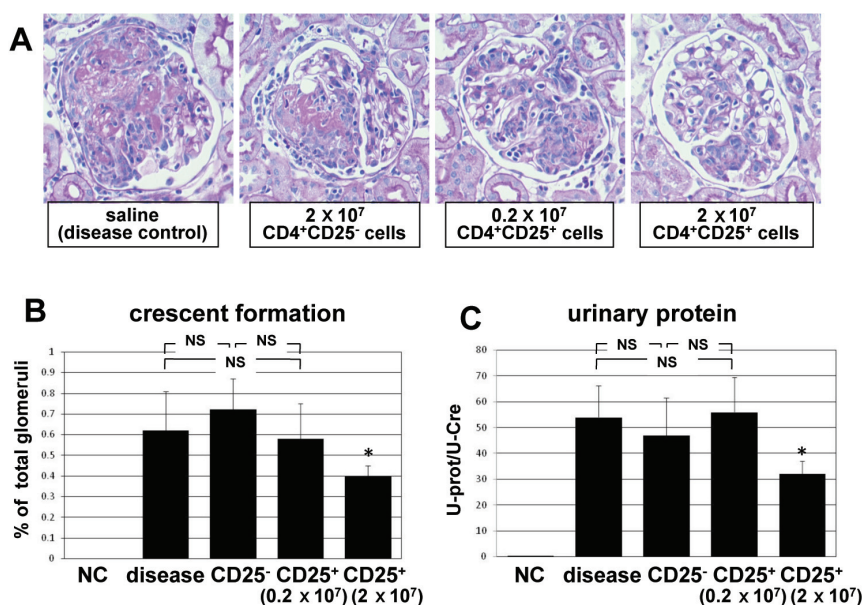


Figure 7. Effect of adoptive transfer of JJ316-induced CD4⁺CD25⁺ T cells into nephritic rats. The experiment was performed according to experimental protocol 2. (A) Representative images of PAS-stained sections of nephritic glomeruli are presented. Original magnification 1,000 \times . (B) Proportions of crescentic glomeruli per total glomeruli. Approximately 120 glomeruli from four rats were assessed in each group. (C) Urinary protein (U-prot) levels per creatinine (U-Cre) level were calculated in each group. Data were presented as means \pm SD. * P < 0.05 versus disease control (saline). NC, normal healthy control.

into rat induces polyclonal B-cell proliferation and thereby elevation of serum titers of IgM, IgG1, IgG2a and IgE (12). This effect was also shown to be indirectly caused by CD4⁺ T-cell activation, since it was absent in athymic rats. Elevation of serum titers of these immunoglobulins are also confirmed in this experiment (data not shown), which may lead to stronger depositions of rat IgG on the GBM.

Elucidating how Treg cells work in this model is of crucial importance. Treg cells have been proposed to use various potential suppression mechanisms, including suppression by inhibitory cytokines (for example, IL-10, transforming growth factor [TGF]- β), suppression by cytotoxicity mediated by granzymes, suppression by metabolic disruption (for example, IL-2 deprivation-mediated apoptosis) and suppression by modulating function of dendritic cells and/or macrophages (23–25). Although the precise molecular mechanism underlying the therapeutic

effect of JJ316 in this model remains elusive, our current data demonstrate that CD4⁺ T-cell-driven responses, especially the propensity of Th1-biased responses to direct crescentic patterns of injury, was reversed by administration of JJ316. First, Th1 cytokines (IFN γ) were downregulated in the glomeruli (data not shown) and in LNs (see Figure 6) after treatment, whereas Th2 (or Treg) cytokines (IL-4 and IL-10) were upregulated. Second, the expression of T-bet, a key transcription factor in the development and homeostasis of Th1 cells lineage (26), was downregulated in LNs (see Figure 6) and spleen (data not shown) after treatment, whereas GATA3 (27) and FoxP3 (3), which correspond to Th2 and Treg cell subsets, respectively, were upregulated. This result is in good agreement with our previous data showing that triggering of T-cell proliferation through CD28 induces GATA3 and promotes Th2 differentiation *in vitro* and *in vivo* (28). Third, there was a significant reduction in the

expression of proinflammatory cytokines (IL-6, TNF α and MCP1) from T cells, macrophages and/or intrinsic renal cells in glomeruli, which are potential contributors during effector T-cell-directed injury (18). Fourth, macrophage accumulation, which is known to be induced via CD4⁺ T-cell effector mechanisms, was significantly diminished.

It is of interest that considerable numbers of macrophages infiltrated the glomeruli, even after treatment. Similar phenomena have been observed in the encephalomyelitis model of the Lewis rat treated with JJ316 (16). Recently, new populations of macrophages have been proposed on the basis of different homeostatic activities including host defense, wound healing and immune regulation (29). In line with this argument, we evaluated the glomerular expression of CD163 (ED2; hemoglobin scavenger receptor). CD163 was implicated as a possible marker for “alternatively activated” macrophages, which appear to possess immune-suppressive activity (30). Immunohistochemical analysis (see Figure 4C) revealed that the CD163⁺ subset is significantly increased among CD68⁺ glomerular macrophages after treatment, suggesting that Treg cells may modulate the phenotype of macrophages leading to resolution of glomerulonephritis. These data coincide with a prior report that Treg cells induce alternative activation of human monocytes/macrophages *in vitro* (31) and suggest that the macrophages that infiltrate the glomeruli after JJ316 treatment could also contribute to the prevention of tissue damage, rather than augment it.

One of the most promising results from our long-term experiment is the finding that a single administration of JJ316 prevented the nephritic rats from death for >2 months after induction of nephritis. The expansion of Treg cells evoked by JJ316 peaked 3 days after injection, and approximately 10 days later, it returned to the basal level (32). Considering that heterologous rabbit IgG continues to deposit on the GBM even 2 months after

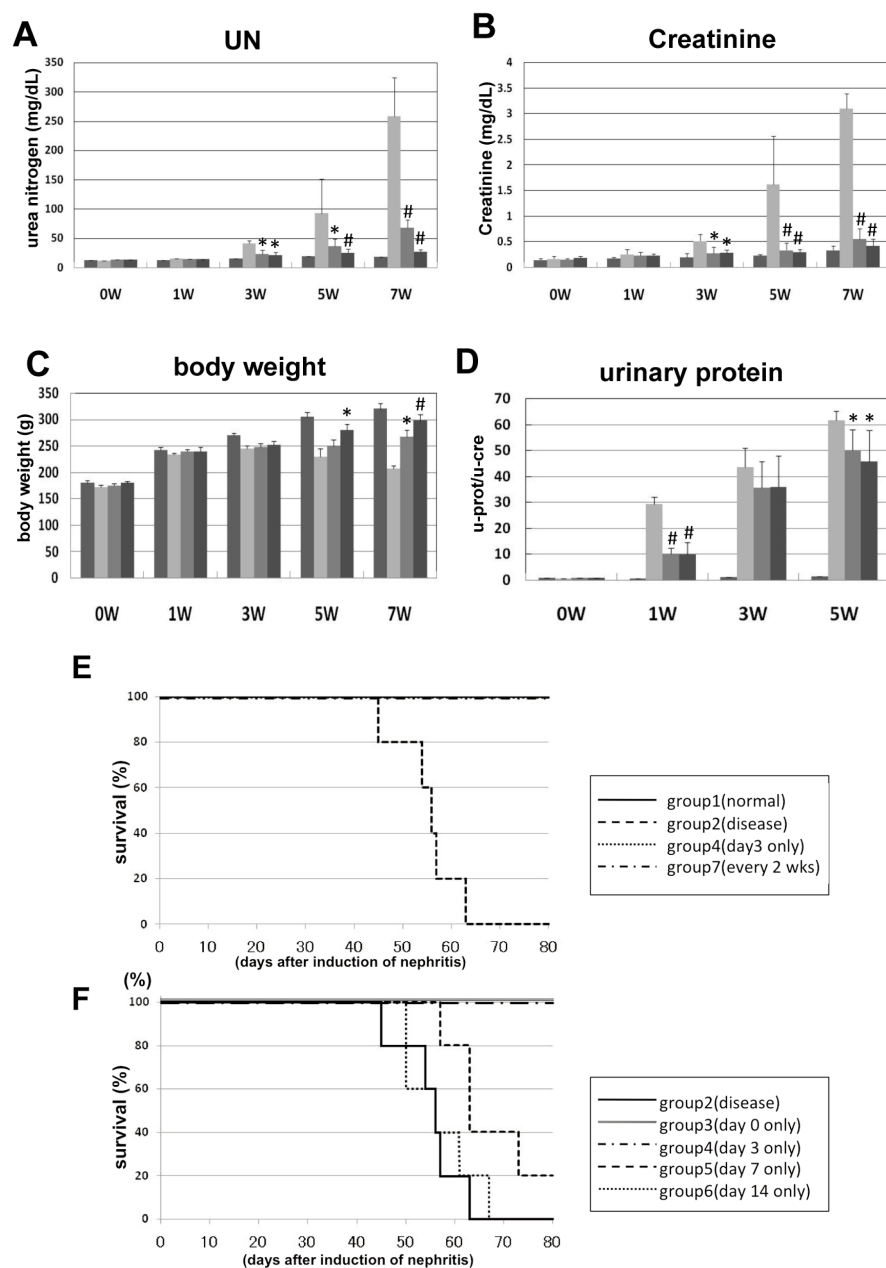


Figure 8. Effect of JJ316 on NTS-induced nephritis in a long-term experiment. The experiment was performed according to experimental protocol 3 (also see Figure 1). (A–D) Serum urea nitrogen (UN) (A), serum creatinine (B), body weight (C) and urinary protein (u-prot) levels (D) (as assessed by the ratio to urinary creatinine (u-cre) concentration) of rats in groups 1, 2, 4 and 7 are shown. (E, F) Survival curves of rats in groups 1, 2, 4 and 7 (E) and in groups 2–6 (F). Data were presented as means \pm SD. * $P < 0.05$ versus disease control. # $P < 0.01$ versus disease control.

disease induction (data not shown) and that therefore antigen stimulation is thought to continue during this period, it is quite plausible that some of the expanded Treg cells differentiated into long-lived memory-type cells and re-

tained immunosuppressive capacity against pathogenic effector T cells after clonal contraction, although further study is required to prove this hypothesis.

In our long-term experiment, the rats treated on day 14 only (group 6) seemed

to have no therapeutic effect. Because most patients of rapidly progressive glomerulonephritis present weeks to months after the onset of disease, the study results could be a concern when future preclinical and clinical studies are planned. However, we have to pay attention to the difference between this rat NTS model and human disease, such as antineutrophil cytoplasmic antibody (ANCA)-associated glomerulonephritis. Crescentic glomeruli were seen as early as on day 7, and rats become uremic ~40–60 days after injection of NTS, which is apparently more progressive than human disease. Therefore, we should not apply data obtained from this rat model directly to human disease.

During our animal experiment, on March 13, 2006, humanized CD28-SA (TGN1412) was administered to six healthy volunteers in London as a phase I clinical trial and caused severe and life-threatening adverse effects owing to the cytokine storm (33,34). The final report prepared by the British Expert Scientific Group on phase I clinical trials reported that the adverse effects did not involve contaminants in the manufacturing of TGN1412 or in its formulation or administration to trial participants (33). It was concluded that the serious adverse events were caused by unexpected biological effects in humans, not predicted by preclinical safety testing.

This life-threatening cytokine release syndrome seems to shatter the hope of translating this promising approach to human therapy. Using a novel mouse antimouse CD28-SA, we reinvestigated the relationship between Treg activation and systemic cytokine release (35). The cytokine storm was not detected in preclinical rodent models because of the rapid control of CD28-SA-induced cytokine release by Treg cells. But depletion of Treg cells before CD28-SA stimulation led to systemic release of proinflammatory cytokines, indicating that in rodents, Treg cells effectively suppress the inflammatory response. Because the human volunteers of the TGN1412 study were not protected by this mechanism, we also

tested whether corticosteroid prophylaxis would be compatible with CD28SA-induced Treg activation. We demonstrated that CD28-SA-induced expansion of Treg cells is not affected by high-dose corticosteroids sufficient to control systemic cytokine release. We hoped it would not only suppress the cytokine storm but also keep the Treg activation, thereby providing an important safety shield in CD28-SA therapies.

In conclusion, we have demonstrated that treatment with CD28-SA has a dramatic therapeutic effect on an experimental crescentic nephritis model, possibly due to an expansion of Treg cells. Effort toward safer therapy that avoids unexpected side effects encountered during the TGN1412 trial will be required to allow successful clinical application of CD28-SA.

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DISCLOSURE

T Hünig is a consultant to TheraMab, the company that now owns TGN1412.

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