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Full title of manuscript

ADDITION OF ANGIOTENSIN II TYPE 1 RECEPTOR BLOCKER TO CCR2 ANTAGONIST MARKEDLY ATTENUATES CRESCENTIC GLOMERULONEPHRITIS

Author's full names and affiliations

Maki Urushihara, MD, PhD, Naro Ohashi, MD, PhD, Kayoko Miyata, PhD, Ryousuke Satou, PhD, Omar W. Acres, MS, Hiroyuki Kobori, MD, PhD

Department of Physiology, and Hypertension and Renal Center of Excellence,

Tulane University Health Sciences Center, New Orleans, LA

Short title

CCR2 and Ang II Receptor on Crescentic Nephritis

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Corresponding author

Hiroyuki Kobori, MD, PhD, FAHA, FASN, FACP

Associate Professor of Departments of Medicine and Physiology

Director of the Molecular Core in Hypertension and Renal Center of Excellence

Tulane University Health Sciences Center

1430 Tulane Avenue, #SL39/M720, New Orleans, LA 70112-2699, USA

Tel: +1-504-988-2591, Fax: +1-504-988-0911, E-mail: hkobori@tulane.edu

Detailed Methods

Regents and Materials. Anti-collagen type I antibody was purchased from Chemicon International. Anti-CD68 antibody was from Serotec. Anti-MCP-1, AT1 receptor, TGF-β1 and TβR1 antibodies were from Santa Cruz Biotechnology. Anti-Ang II antibody was from Phoenix Pharmaceuticals. Anti-AGT antibody was from Immuno-Biological Laboratories (IBL). Pan-specific TGF-β neutralizing antibody and TGF-β1 Immunoassay kit were from R and D Systems. Rat MCP-1 ELISA Kit was from Thermo Scientific. A 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) was from Roche Applied Science. A Sircol collagen assay was from Biocolor. ARB of olmesartan was kindly provided by Daiichi Sankyo.

Kidney Histology and Immunohistochemistry

Glomeruli are considered to exhibit crescent formation when at least 2 layers of cells are observed in the Bowman space. The number of crescents was determined from 50 glomeruli per rat, and this value was expressed as the mean percentage for each group. The ratio of area occupied by crescents in each glomeruli was estimated and assigned one of the following scores: 0, absent; 1, < ¼; 2, between ¼ and ½; 3, between ½ and ¾; and 4, more than ¾ of the whole glomerulus [1]. The mean score of estimated glomeruli was then calculated as the crescent score. The extent of glomerular fibrotic area was quantitatively evaluated by an automatic image analysis, which determined the area occupied by glomerular staining positively for collagen in Masson's trichrome-stained section (Mass Histology), as described previously [2 3]. Paraffin-embedded kidney tissues were sectioned, deparaffinized, and incubated with either antibody. Immunohistochemistry was performed by a robotic system (Dako) as previously described [4 3]. The fraction of fibrotic (blue) or immuno-reactive area (brown) in the glomeruli was measured using the Image-Pro Plus software (Media Cybermetics). For each rat, 20 glomeruli were examined and mean percentages of the affected lesions were calculated. CD68-positive cells (brown) were counted in the glomeruli in each microscopic field. The averaged numbers of macrophages/monocytes in the glomeruli were then obtained for each rat.

Quantitative Real-Time RT-PCR

Total RNA was extracted using a commercially available kit (Qiagen) and subjected to DNase 1 treatment (Invitrogen) to eliminate contaminant genomic DNA. Quantitative real-time RT-PCR was performed as previously described [3, 4]; the data thus obtained were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression levels. The PCR primers used in this study are listed in Supplemental Table S1.

Western Blot Analysis

Protein extraction and western blot analysis were performed on samples using an infrared imaging system (LI-COR Biosciences), as described [3]. Appropriate secondary antibodies were purchased from LI-COR.

Rat Parietal Epithelial Cell (PCE) culture

Glomerular PECs were cultured using a previously method [5], with some modification. Glomeruli were isolated from rat kidney by multiple sieving techniques and placed under an inverted tissue culture microscope with phase-contrast optics. Encapsulated glomeruli were selected from tissue suspension by sucking into a micropipette and placed on collagen-coated culture dish. The cultured PECs were out growths from glomeruli and were polygonal, with a cobble-like appearance. Cell clones were proliferated in K1-3T3 medium.

Cytokine Determination by ELISA

We carried out sandwich ELISAs for rat MCP-1 or TGF-β1 with supernatants form MCs in accordance with the manufactures' specifications. MCs were seeded in completed medium into

12-well plate. Twenty-four hours later, cells were washed with PBS and then covered with serum free medium for additional 24 h. For measurement of total TGF-β1, supernatants were acidified with 1 N HCl for 10 min and then neutralized with 1.2 N NaOH right before application.

Cell Proliferation Assay and Collagen Measurement

Cell proliferation ability was evaluated by the WST-1 assay according to the manufacturer's protocol. Briefly, co-cultured cells were grown to sub-confluent in medium containing serum, and subsequently starved for 24 h. Then, cells were cultured for 24 h with or without treatment, and WST-1 reagent was added to cell culture medium in each well. After incubation for 4 h, the absorbency of the samples was measured with a microplate reader at wavelength of 450 nm [6 7].

The soluble collagen levels in culture supernatants were measured by using a Sircol collagen assay after incubation for 24 h. This assay measured total secreted collagen from cultured cells. One milliliter of Sirius red, an anionic dye that specifically reacts with basic side chain groups of collagens, was added to the supernatant and incubated with gentle rotation for 30 min at room temperature. After centrifugation, the collagen-bound dye was re-solublized in 0.5 M NaOH, and the absorbance at 540 nm was measured [6].

References

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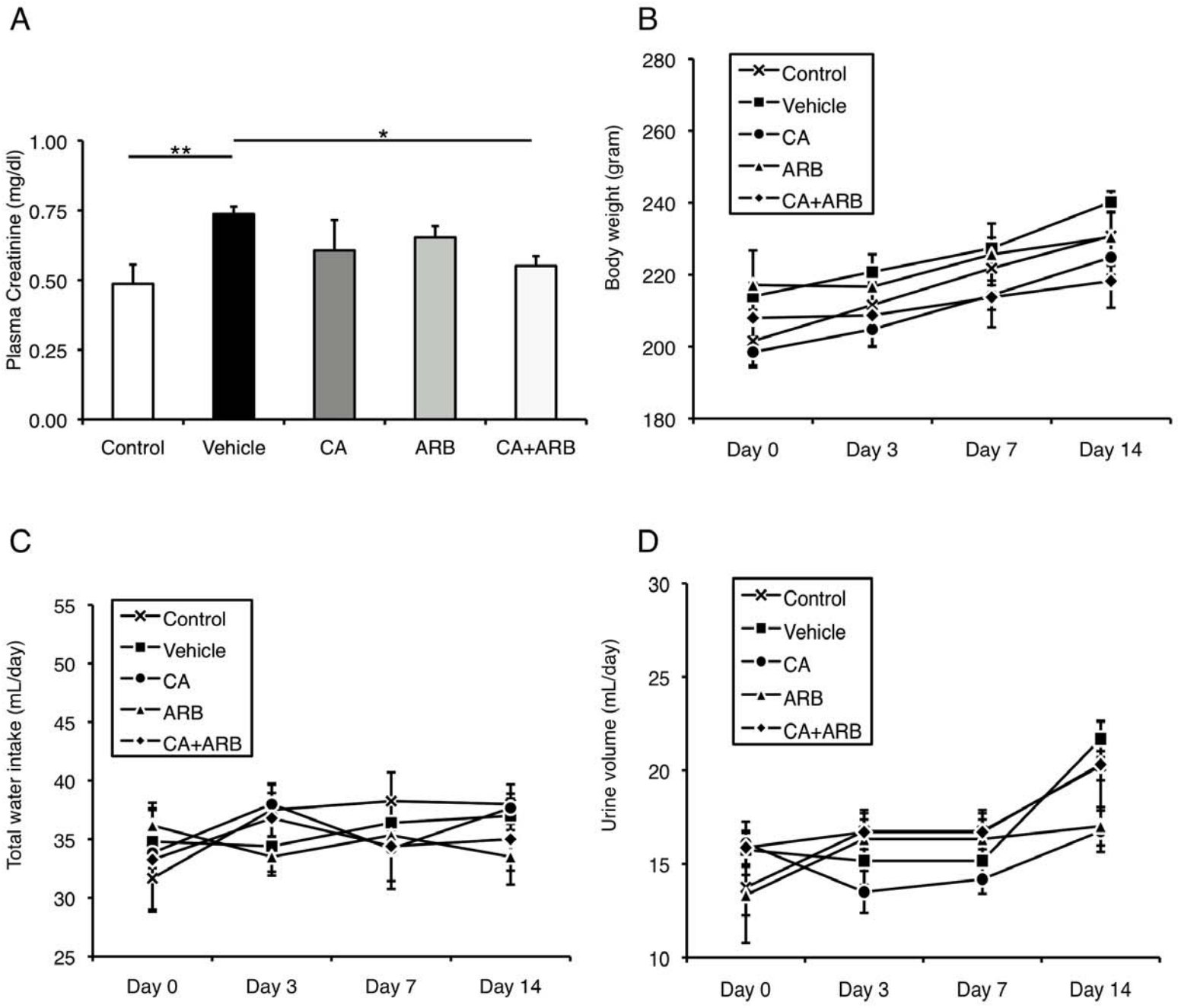
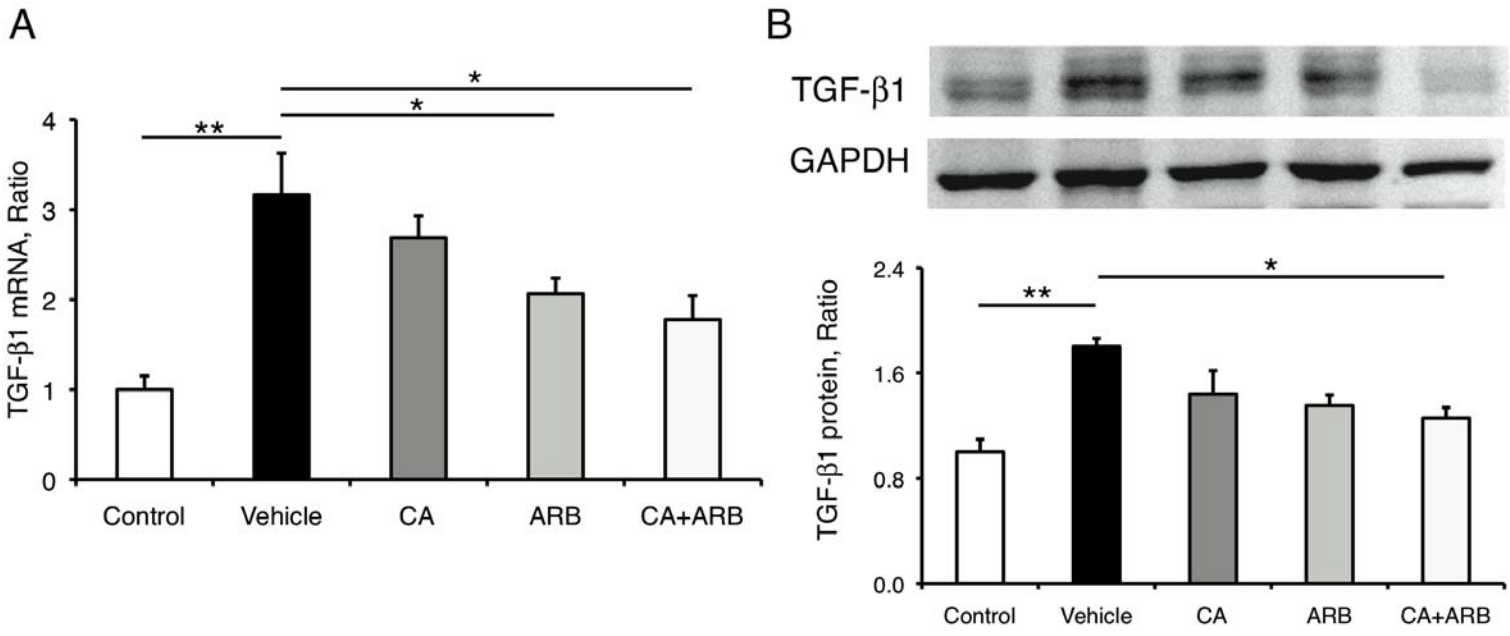
Supplemental Table S1

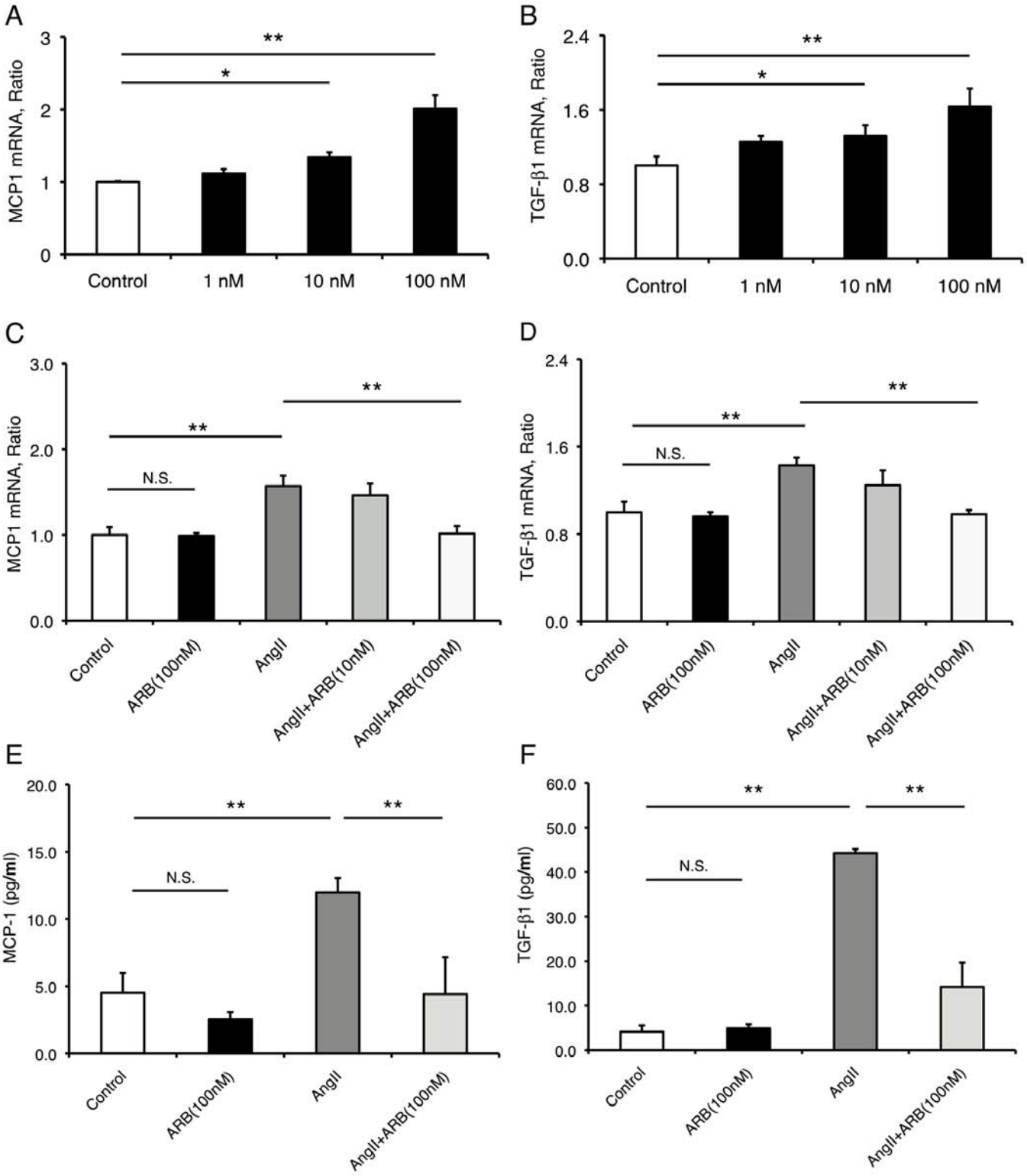
Gene	Sense Primer	Antisense Primer	Probe
GAPDH	CAGAACATCATCCCTGCATC	CTGCTTCACCACCTTCTTGA	CCTGGAGAAACCTGCCAAGTATGATGA
MCP-1	AGCACCTTTGAATGTGAACT	AGAAGTGCTTGAGGTGGTT	CCCATAAATCTGAAGCTAATGCATCC
TGF-β1	TACCATGCCAACTTCTGTC	AAGGACCTTGCTGTACTGTGT	CCCTACATTTGGAGCCTGGAC
Collagen type1	GCATCAAGGTCTACTGCAACA	CAGACATGCTTCTTCTCCTT	CCAGAAGAACTGGTACATCAGCCCA



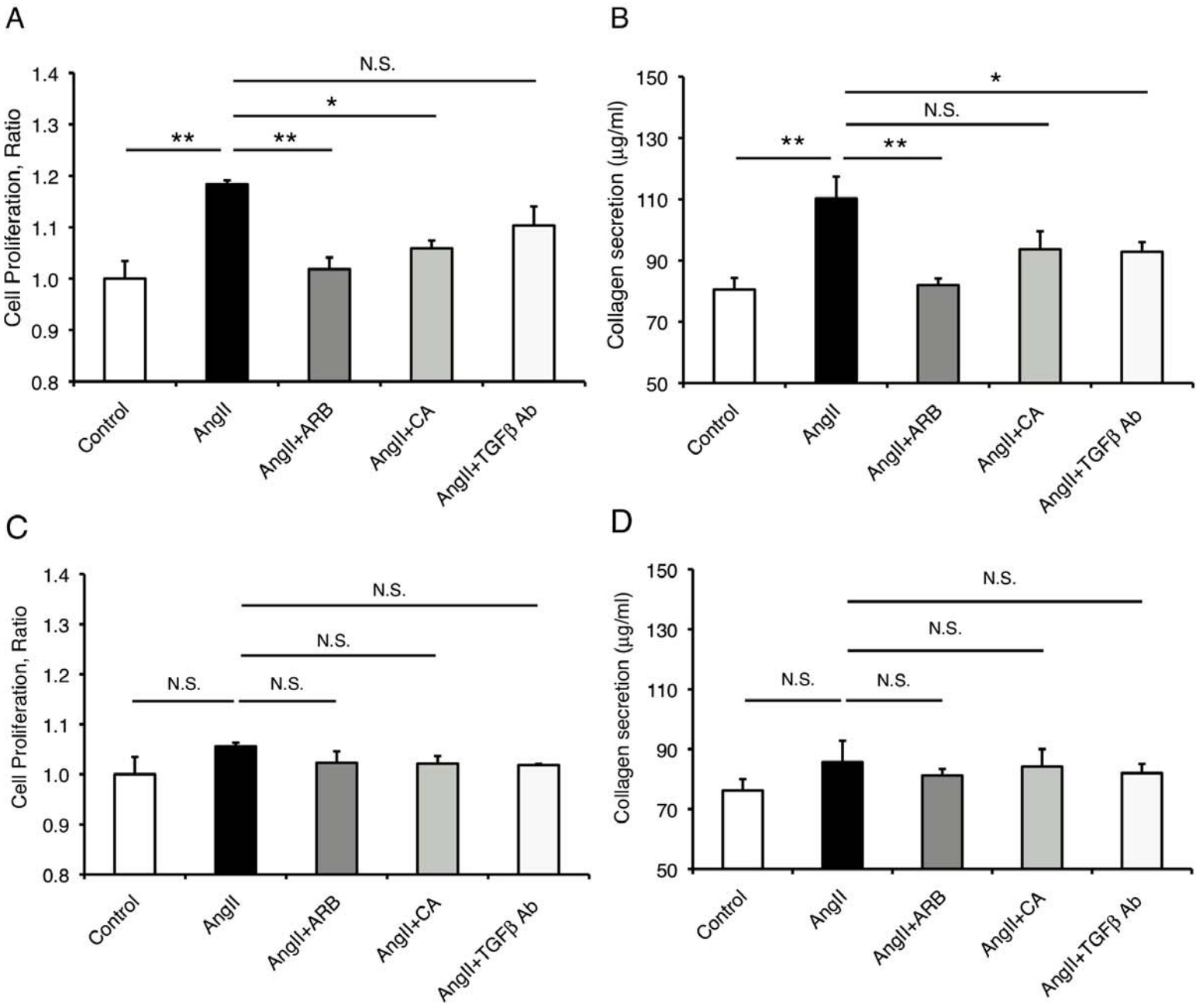
Supplemental Figure S1. The level of plasma creatinine, body weight, total water intake, and daily urine volume in anti-GBM disease rat model. (A) The level of plasma creatinine, (B) body weight, (C) total water intake, and (D) daily urinary volume. Data are mean +/- SEM. * $P < 0.05$, and ** $P < 0.01$ between groups as indicated.



Supplemental Figure S2. Effects of the treatment on TGF-β1 expression in anti-GBM disease rat model. (A) Quantitative real-time RT-PCR of TGF-β1 mRNA in the isolated glomerulo of all tratment groups. (B) Western blot analyses of TGF-β1 protein levels in isolated glomeruli of all treatment groups. Data are mean +/- SEM. * $P < 0.05$, and ** $P < 0.01$ between groups as indicated.



Supplemental Figure S3. Effects of angiotensin II (Ang II) on cultured mesangial cells (MCs). (A and B) mRNA levels of MCP-1 (A) and TGF-β1 (B) in cultured MCs. MCs were stimulated with Ang II for indicated concentrations and analyzed by quantitative real-time RT-PCR. (C and D) mRNA levels of MCP-1 (C) and TGF-β1 (D) in cultured MCs. MCs were pretreated with Ang II type 1 receptor blocker (ARB) and subsequently stimulated with 100 nM Ang II. Sandwich ELISA for secretion of MCP-1 (E) and TGF-β1 (F) in MCs. MCs were pretreated with ARB (100 nM) and subsequently stimulated with 100 nM Ang II. Data are mean +/- SEM. ** $P < 0.01$ between groups as indicated. N.S., not significant.



Supplemental Figure S4. Effects of angiotensin II (Ang II) on cultured mesangial cells (MCs) and cultured parietal epithelial cells (PECs) co-cultured with macrophages with/without MCs. (A) Cell proliferation in PECs co-cultured with macriphages and MCs using WST-1 assay. (B) Collagen secretion in PECs co-cultured with macrophages and MCs using Sircol assay. (C) Cell proliferation in PECs co-cultured with macrophages without MCs using WST-1 assay. (D) Collagen secretion in PECs co-cultured with macrophages without MCs using Sircol assay. PECs co-cultured with macrophages were pretreated with ARB (100 nM), CCR2 antagonist (CA, 10 μM) or pan-specific TGF-β neutralizing antibody (TGFβ Ab, 10 μg/ml) and subsequently stimulated with 100 nM Ang II. Data are mean +/- SEM. ** $P < 0.01$ between groups as indicated. N.S., not significant.