

博士論文

Vagus nerve stimulation even after injury ameliorates cisplatin-induced nephropathy via reducing macrophage infiltration

(迷走神経刺激は障害後であっても腎保護効果を有する)

宇仁 理恵

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東京大学大学院 医学系研究科 内科学専攻 博士課程

腎臓・内分泌内科学

指導教員 南学 正臣 教授

宇仁 理恵

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Abstract

The efficacy of prior activation of an anti-inflammatory pathway called the cholinergic anti-inflammatory pathway (CAP) through vagus nerve stimulation (VNS) has been reported in renal ischemia-reperfusion injury models. However, there have been no reports that have demonstrated the effectiveness of VNS after injury. I investigated the renoprotective effect of VNS in a cisplatin-induced nephropathy model.

C57BL/6 mice were injected with cisplatin, and VNS was conducted 24 hours later. Kidney function, histology, and a kidney injury marker (Kim-1) were evaluated 72 hours after cisplatin administration. To further explore the role of the spleen and splenic macrophages, key players in the CAP, splenectomy, and adoptive transfer of macrophages treated with the selective $\alpha 7$ nicotinic acetylcholine receptor agonist GTS-21 were conducted.

VNS treatment significantly suppressed cisplatin-induced kidney injury. This effect was abolished by splenectomy, while adoptive transfer of GTS-21-treated macrophages improved renal outcomes. VNS also reduced the expression of cytokines and chemokines, including CCL2, which is a potent chemokine attracting monocytes/macrophages, accompanied by a decline in the number of infiltrating macrophages.

Taken together, stimulation of the CAP protected the kidney even after injury in a cisplatin-induced nephropathy model. Considering the feasibility and anti-inflammatory effects of VNS, the findings suggest that VNS may be a promising therapeutic tool for acute kidney

injury.

Introduction

Despite the advancements in modern medical technology, acute kidney injury (AKI) is still one of the major comorbidities in hospital settings. It is estimated that AKI occurs in approximately 15% of hospitalized patients and 60% of critically ill patients [1], and morbidity and mortality rates remain high [2,3]. In addition, AKI is a risk factor for chronic kidney disease (CKD) and end-stage renal disease (ESRD) [4]. Therefore, prevention of AKI development and progression to CKD is essential. However, despite of the innovations in medicine, supportive and preventative methods such as hydration or avoidance of renal toxic drugs remain the main treatment options for AKI. No safe and effective therapeutic modalities for AKI have been established to date.

Inflammation plays an important role in the pathogenesis of AKI [5]. Moreover, chronic inflammation contributes to the progression of CKD. Therefore, suppression of inflammation plays a potential role in treating kidney injury.

Recently, a new anti-inflammatory pathway called the cholinergic anti-inflammatory pathway (CAP) has been discovered [6]. This anti-inflammatory pathway was first described by Dr. Tracey's group using endotoxemia model in 2000 [7]. They showed that acetylcholine reduced tumor necrosis factor (TNF) secretion from human macrophages after lipopolysaccharide (LPS) administration and that efferent vagus nerve stimulation significantly decreased serum TNF and liver TNF expression levels in LPS treated rats. The

CAP consists of both afferent and efferent arms, and both afferent and efferent vagus nerves play important roles. The afferent vagus nerve conducts inflammatory information from the peripheral organs to the central nervous system. In the brainstem, the afferent vagus nerve activates the C1 neurons, which make a major contribution to the central regulation of autonomic function [8], and further stimulate the efferent vagus nerve [9] (Figure 1).

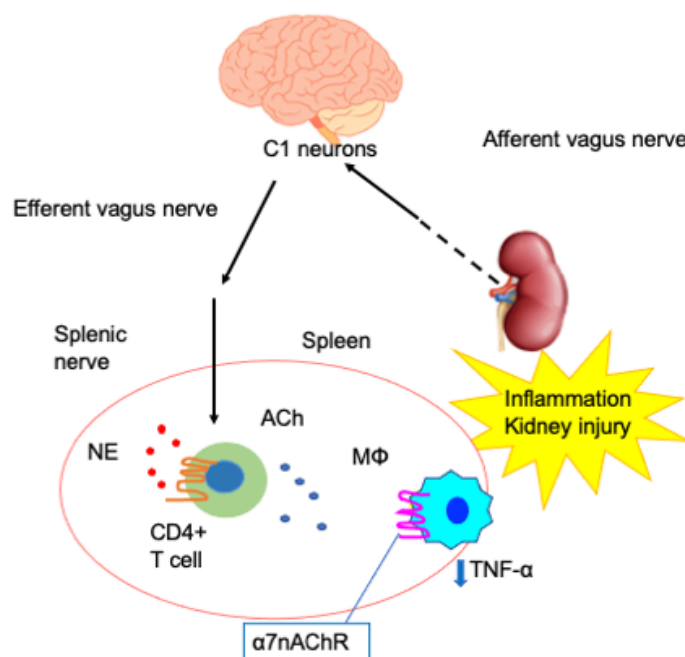


Figure 1. Cholinergic anti-inflammatory pathway (CAP).

Afferent vagus nerve conveys inflammatory information from peripheral organs including the kidney to the central nervous system. In the brainstem, C1 neurons are stimulated by afferent vagus nerve and stimulate efferent vagus nerve. Although exact mechanism is still to be elucidated, the splenic nerve is activated by efferent vagus nerve and release norepinephrine (NE) to stimulate CD4 positive T cells in the spleen. Followingly these T cells release acetylcholine (ACh) and act on alpha 7 nicotinic acetylcholine receptor ($\alpha 7nAChR$) positive macrophages ($M\phi$), exerting anti-inflammatory effects by suppressing inflammatory cytokines including $TNF-\alpha$.

Previously, Inoue and Abe et al. reported that vagus nerve stimulation (VNS) protects the kidney from ischemia-reperfusion injury (IRI), which is a well-established

animal sterile kidney injury model, through activation of the CAP [10]. In their study, they performed VNS 24 hours before IRI, and evaluated kidney function 24 hours after IRI. VNS led to reno-protection, decreasing plasma creatinine level and kidney Kim-1 expression level with attenuated tubular injury. Another interesting finding of the same study is that, although VNS applied 10 minutes prior to IRI failed to confer kidney protection, VNS at 24–48 hours before IRI provided a significant and long-lasting renoprotective effect that was maintained for at least 48 hours. These findings suggest that there is a delay between VNS and the establishment of VNS-induced renoprotection, but that, once established, the protective effect is maintained without continuous stimulation. Given the rather instantaneous property of neural conduction, there seems to be a complex immunological mechanism. Although there are many kinds of inflammatory cells such as B cells, T cells, and dendritic cells in the spleen, the anti-inflammatory effect of CAP stimulation is delivered through activation of $\alpha 7$ nicotinic acetylcholine receptor ($\alpha 7$ nAChR) on splenic macrophages [11]. Considering its anti-inflammatory effect, VNS is a potent tool for treating inflammatory disorders such as sepsis, lung injury, rheumatoid arthritis, inflammatory bowel disease, and diabetes [12]. VNS was approved by the Food and Drug Administration in 1997 for the treatment of refractory partial-onset epilepsy [13] and in 2005 for chronic or recurring depression [14]. The VNS device is a battery-powered apparatus akin to a cardiac pacemaker. In most models, stimulating electrical leads are surgically implanted in the carotid sheath around the left

vagus nerve. Its safety is proven in that more than 100,000 VNS devices have been implanted in over 75,000 patients worldwide [15]. Yet, it is still an invasive surgery, and therefore the device cannot be implanted for all hospitalized patients. In addition, VNS-related side effects such as pain, temporal facial paresis or even irreversible nerve damage have been reported [16]. Accordingly, noninvasive VNS methods have been developed. One of them is transcutaneous ultrasound, which is proven effective by animal models, and a clinical trial is on-going to test its effectiveness for rheumatoid arthritis [17]. The other option is transcutaneous VNS devices [18,19], wherein the vagus nerve is stimulated via the auricular branch of the nerve by small, earphone-like electrodes. A pilot study demonstrated that noninvasive VNS downregulated inflammatory cytokine release in healthy subjects [19]. These results reinforce the fact that VNS is safe and readily applicable in hospital settings. Given the high prevalence of AKI and the difficulty of identifying patients who will develop AKI, it would be more beneficial if VNS has therapeutic effect, even after injury.

IRI is characterized by the robust inflammatory responses, which starts from the initial ischemic insult and is accelerated by following reperfusion phases (Figure 2) [20]. Post ischemia, proinflammatory damage-associated molecular patterns (DAMPs), hypoxia-inducible factors (HIFs) and adhesion molecules play important roles in recruitment and activation of various immune cells [21]. Specifically, following ischemia injury, kidney cells

release DAMPs. These DAMPs activate toll-like receptors expressed on kidney tubular cells, subsequently activating complement system and cytokine production leading to leukocyte recruitments. Neutrophils, macrophages and natural killer (NK) cells infiltrate in the kidneys within several hours after injury. In reperfusion phase, delivery of oxygen to previously ischemic cells promotes formation of reactive oxygen species (ROS) [22].

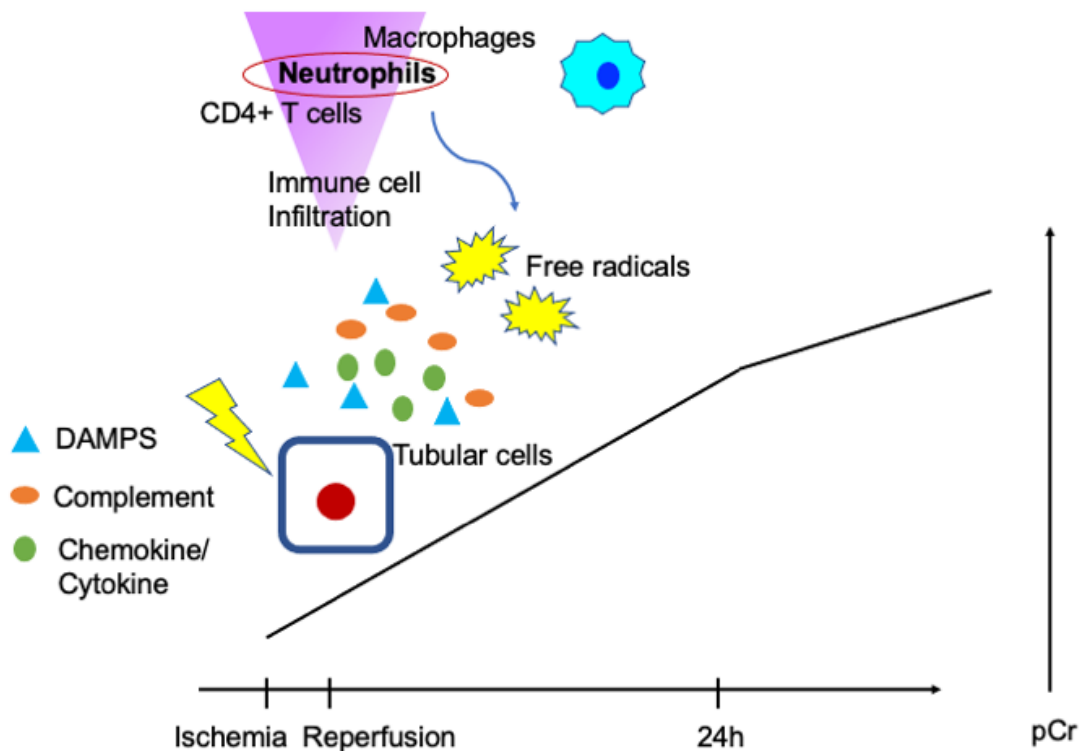


Figure 2. Mechanism of ischemia-reperfusion injury (IRI) in the kidney.

Following ischemia, damage-associated molecular patterns (DAMPs) and cytokines are released from injured cells and inflammatory cells infiltrate into the kidney. Free radicals generated from neutrophils further damage cells. Plasma creatinine (pCr) level is already elevated 24 hours after ischemia injury.

Another well-established aseptic acute kidney injury animal model is cisplatin-induced nephropathy. Cisplatin is a major tumoricidal drug that has long been used for the

treatment of a number of cancers. Cisplatin induces AKI, an important dose-limiting toxicity that frequently leads to cessation of therapy [23]. Cisplatin is filtered by the kidney glomeruli and also secreted from tubular cells. And its concentration is higher in the kidney than in blood, making the kidney susceptible to cisplatin-associated side effects [24]. In tubular cells, cisplatin uptake is mediated by basolateral organic cation transporters (OCTs) and secreted from apical efflux transporters [25]. The mechanism underlying tumoricidal effect of cisplatin is believed to cause DNA damage. First, it binds to DNA and forms inter-strand cross-links, leading to the cassation of the DNA synthesis. Additionally, mitochondrial DNA damage also contributes to the cisplatin-induced cellular damage. In cisplatin-induced nephropathy, DAMPs are also released from injured tubular epithelial cells, activating TRLs followed by the secretion of inflammatory cytokines and chemokines. Neutrophils and macrophages infiltrate into the kidneys after injury, but their roles in the pathogenesis of kidney injury are yet to be described (Figure 3).

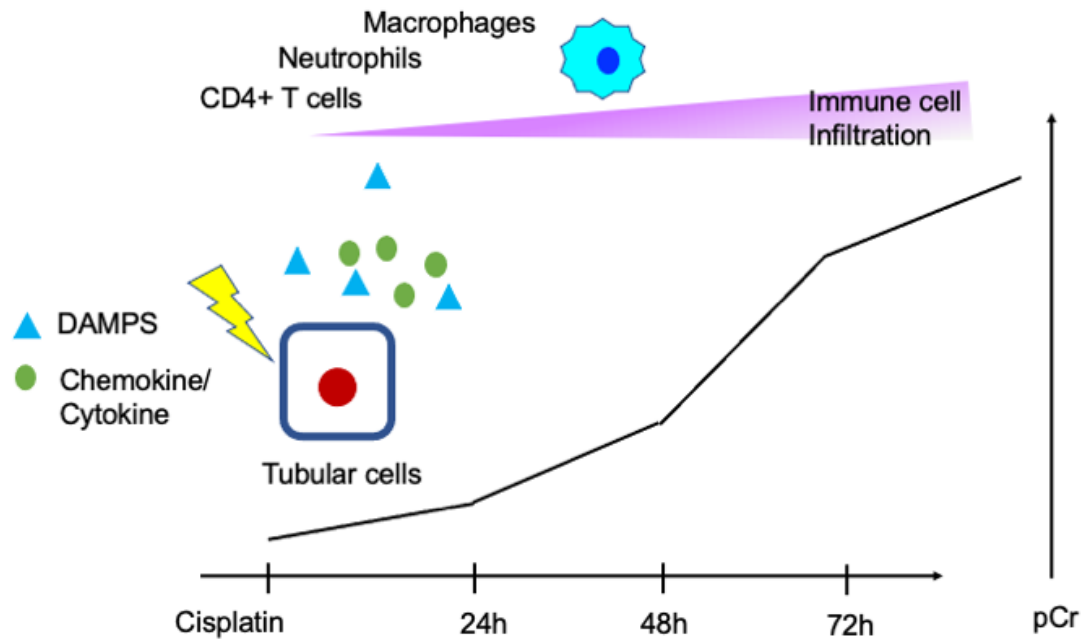


Figure 3. Mechanism of cisplatin-induced nephropathy.

Damaged tubular epithelial cells secrete DAMPs, activating TLRs. Subsequently, cytokines and chemokines are released and attract inflammatory cells. Plasma creatinine levels gradually increase and reach their highest at around 72 hours.

DAMPs, damage associated molecular patterns; TLRs, toll-like receptors

In this study, I hypothesized that VNS is renoprotective after the development of AKI, and I investigated this using an IRI model and a cisplatin-induced nephropathy model both.

Methodology

Animals

C57BL/6 male mice (7–10 weeks old, 20–25 g) were purchased from Sankyo Labo Service Corporation (Tokyo, Japan). All procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals or the equivalent, and the procedures were approved by the Ethics Committee for Animal Care and Use of The University of Tokyo, Tokyo, Japan.

All surgeries and euthanasia were performed under general anesthesia (medetomidine 0.3 mg/kg, butorphanol 5 mg/kg, and midazolam 4 mg/kg).

Bilateral renal ischemia reperfusion injury (IRI)

Animals were placed on the heating pad to maintain body temperature around 35.5°C, and body temperature was monitored using rectal thermometers. After shaving hairs on the back, flank incisions were placed on both sides. Renal pedicles were clamped for 26 minutes with metal clips as reported [26]. The clamps were released, and the wound was sutured after blood flow was restored. Sham-operated mice underwent the same procedure except for renal pedicle clamps. Kidney function was evaluated 24 hours after bilateral IRI.

Vagus nerve stimulation

The left cervical vagus nerve was stimulated as previously reported [10] using an

Isostim Stimulator (A320RC; World Precision Instruments, Sarasota, FL, USA). I isolated the left vagus nerve by mid-cervical incision and placed bipolar silver electrodes (AS633; Cooner Wire) (Figure 4). Electrical stimulation (frequency, 5 Hz, square wave; 50 μ A; duration, 1 ms) was applied for 10 minutes to the VNS group. High frequency (20-30 Hz) stimulation is effective for refractory epilepsy and depression, whereas low frequency (5 Hz) is used for CAP activation and inflammatory diseases including sepsis, burn-induced intestinal barrier injury and colitis [27]. Since more side effects such as cough, hoarseness and paresthesias appear in humans with higher frequency, I decided to adopt 5 Hz expecting anti-inflammatory effects of VNS. In the previous report, Dr. Inoue et al. optimized VNS protocol using real-time vital sign monitoring system. They stimulated the vagus nerve with three different currents (10, 30 and 50 μ A). There was no significant difference in systemic blood pressure change, which is one of the important factors for kidney function, in these groups, while stimulation with 50 μ A significantly decreased heart rate suggesting the vagus nerve is effectively stimulated. Although heart rate was decreased significantly, the change was small (around 10 beat per minute [bpm]) compared to normal heart rate (around 500 bpm) and it had little impact on hemodynamics. They also showed that VNS (frequency, 5 Hz, square wave; 50 μ A; duration, 1 ms) 24 hours before LPS administration successfully decreased plasma TNF concentration in mice, confirming its anti-inflammatory effect. Unfortunately, I lacked the real-time monitoring system for mice, so I adopted the same

parameters for this research. For the sham operation, I simply exposed the vagus nerve using an identical incision. After the operation, the anesthesia was reversed with the $\alpha 2$ -adrenergic receptor antagonist, atipamezole (0.5 mg/kg). VNS or sham operation were applied to mice 24 hours after cisplatin injection, and 48 hours after VNS or sham operation, kidney functions were evaluated.

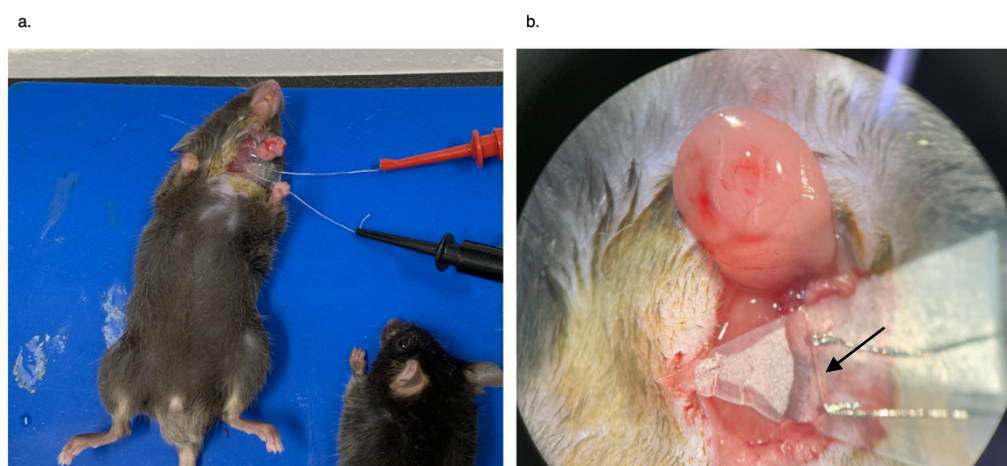


Figure 4. Vagus nerve stimulation on mice.

(a) Electrodes are placed on left vagus nerve. (b) A black arrow indicates the left vagus nerve.

Cisplatin induced nephropathy mouse model

Cisplatin (25 mg/kg) dissolved in 0.9% normal saline was administered by intraperitoneal injection, and the mice were euthanized 24 or 72 hours later under anesthesia by cervical dislocation. The vehicle-treated group received the same volume of normal saline intraperitoneally.

Systemic administration of GTS-21, a selective agonist of $\alpha 7$ nAChR

Mice were divided into 4 groups: control and GTS-1, 2 and 4. GTS-1 received GTS-21 (20 µg/g) once 24 hours after cisplatin administration. GTS-2 received the drug (20 µg/g) every 24 hours after cisplatin injection. GTS-4 received reduced dose (10 µg/g) twice a day for the following 2 days.

Splenectomy

Five days prior to cisplatin injection, splenectomy was conducted under general anesthesia. The splenic arteries and veins were ligated, and the spleen was removed through a small left back incision. In sham-operated mice, the spleen was just exposed.

Adoptive transfer of macrophages

Spleens were collected from wild-type donor mice and strained to single-cell suspensions through 40 µm filters with sterile PBS. 1×10^8 cells were labeled with anti-F4/80 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany), and F4/80 positive splenocytes were selected using the magnetic cell separation method (MACS). The cells were incubated with either 100 µM GTS-21, a selective agonist of $\alpha 7$ nAChR, or vehicle for 1 hour and were washed twice with PBS. An injection of 1×10^5 macrophages diluted with 200 µL of normal saline was administered intravenously (Figure 5).

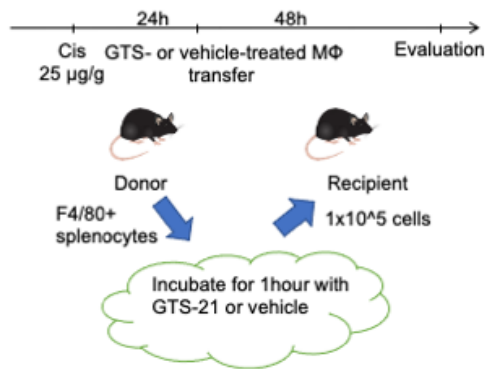


Figure 5. A schematic depiction of adoptive macrophage transfer.

1×10^5 splenic macrophages were transferred from donor mice to recipients.

Evaluation of kidney function

Blood samples were centrifuged at 7,000 g for 5 minutes and plasma was collected.

Plasma creatinine level was measured with an enzymatic method based on the manufacturer's protocol (Wako Pure Chemical Industries, Saitama, Japan). Blood urea nitrogen (BUN) level was measured by SRL Inc. (Osaka, Japan).

Immunohistochemistry

Whole kidneys were cut horizontally into four parts, and one of the central parts was fixed in Mildform 10N (Wako Pure Chemical Industries) before being embedded in paraffin. Tissue sections (3 μm) were stained with periodic acid-Schiff for evaluation of tubular damage. The tubulointerstitial injury score was graded (0–4) blindly by Prof. Inagi as previously reported [28]. Semiquantitative scores of tubular injury were graded based on the proportion of injured tubules as follows: none = 0; <25% = 1; 25%–50% = 2; 50%–75%

= 3; and >75% = 4. Four fields in the outer medulla were selected randomly for each sample, and the average score was calculated.

Expression of Kim-1, which is known to be upregulated early before the elevation of plasma creatinine in cisplatin-induced nephropathy [29], was assessed by immunostaining using Mouse TIM-1/KIM-1/HAVCR Antibody #AF1817 (R&D Systems, Inc., Minneapolis, MN, USA, diluted 1:200). Embedded tissue was first deparaffinized in Histo-clear (Cosmo Bio, Tokyo, Japan) and rehydrated with ethanol. Sections were boiled in sodium citrate (10 mM, pH 6.0) for 10 minutes using a microwave oven. The sections were blocked with 3% H₂O₂ for 15 minutes and Protein Block (Agilent Technologies, Inc., Santa Clara, USA) for 10 min at room temperature. A VECTASTAIN Elite ABC-HRP Kit, Peroxidase (Goat-IgG) - (PK-4005) (Vector Laboratories, Burlingame, CA, USA) were used for the following steps. The signal was detected using ImmPACT DAB Substrate, Peroxidase (HRP) - (SK-4105) (Vector Laboratories). The percentage of positive signal area was analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

For immunostaining of F4/80 positive macrophages, kidneys were fixed in methyl Carnoy's solution (60% methanol, 30% chloroform, and 10% acetic acid). Renal macrophages were stained using rat anti-mouse F4/80 antibody MCA497 (Cl: A3-1) (Bio-Rad Laboratories, Hercules, CA, USA; diluted 1:400) and subsequent incubation with Histofine Simple Stain Mouse MAX-PO (Rat) (Nichirei Biosciences Inc., Tokyo, Japan).

The number of infiltrating macrophages was counted as the mean value of three randomly selected areas.

RNA isolation and quantitative real-time polymerase chain reaction (PCR)

Renal mRNA was isolated from the edge of one of the slices from the left kidneys using RNAiso Plus (Takara Bio Inc., Shiga, Japan). For RNA isolation from cells, I used a RNeasy Mini Kit (QIAGEN, Venlo, Netherlands). Reverse transcription was performed using PrimeScript RT master mix (Takara Bio). The cDNA was then used to determine relative mRNA expression with Fast SYBR Green Master Mix (Applied Biosystems, Waltham, MA, USA) on a StepOnePlus Real-Time PCR System (Applied Biosystems). Relative expression was calculated using the comparative cycle threshold (CT; $2^{-\Delta\Delta Ct}$) method. Primer sequences are listed in Table 1.

Table 1. Primer list

Kim-1 Fw	mouse	ACATATCGTGGAATCACAACGAC
Kim-1 Rv	mouse	ACTGCTCTTCTGATAGGTGACA
GAPDH Fw	mouse	AGGTCGGTGTGAACGGATTTG
GAPDH Rv	mouse	TGTAGACCATGTAGTTGAGGTCA
CCL11 Fw	mouse	GAATCACCAACAACAGATGCAC
CCL11 Rv	mouse	ATCCTGGACCCACTTCTTCTT
G-CSF Fw	mouse	CAGCCCAGATCACCCAGAATC
G-CSF Rv	mouse	GCTGCAGGGCCATTAGCTTC
IL-12b Fw	mouse	ATTACTCCGGACGGTTCACG
IL-12b Rv	mouse	ACGCCATTCCACATGTCACT
CCL2 Fw	mouse	GACCTTAGGGCAGATGCAGT
CCL2 Rv	mouse	AGCTGTAGTTTTTGTCAACCAAGC
BAX Fw	mouse	AGATGAACTGGACAGCAATATGG
BAX Rv	mouse	GCAAAGTAGAAGAGGGCAACCAC

Cytokine/chemokine immunoassay of plasma

A Bio-Plex Pro Mouse Cytokine GI 23-plex panel was used to determine the plasma cytokine and chemokine levels in the cisplatin-induced nephropathy mouse model. The assay was performed based on the Bio-Plex Pro assay protocol (Bio-Rad). Clustering was performed using Cluster 3.0 [30], and a heatmap was created with Java TreeView 1.1.6r4 [31].

Cell culture

RAW 264.7 murine macrophage cells were procured from the American Type Culture Collection and grown in a Roswell Park Memorial Institute 1640 medium (Sigma-Aldrich) supplemented with 5% fetal bovine serum (FBS; Sigma-Aldrich, St Louis, MO, USA) and

1% penicillin and streptomycin (PS) at 37°C in a 5% CO₂ incubator. HK-2 human proximal tubular cells were obtained from the American Type Culture Collection and grown in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 nutrient mixture (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich).

1×10⁵ cells were plated in 24 wells plate. Cisplatin (5 nM for RAW 264.7 and 20 uM for HK-2) and GTS-21(100 uM) was administered, and the cells were incubated for 24 hours.

Flow cytometry analysis

Kidney suspensions were prepared from mice injected with cisplatin with or without VNS. Kidneys were weighed, minced, and incubated with collagenase (Sigma-Aldrich) and DNase I (Sigma-Aldrich) in RPMI buffer with 10% FBS for 40 minutes at 37°C. The digested kidney tissue suspension was filtered through a 70-µm and 40-µm cell strainer (Greiner Bio-One, Kremsmünster, Austria) via the rubber end of a 2.5-ml syringe plunger and then centrifuged at 500 g for 5 minutes at 4°C. The cells were centrifuged again, the supernatant was discarded, and the cells were resuspended with Flow Cytometry Staining Buffer (Thermo Fisher Scientific, Santa Clara, CA, USA). After blocking nonspecific Fc binding with anti-mouse CD16/32 (2.4G2 ; Thermo Fisher Scientific), fresh kidney suspensions were incubated with the following antibodies: anti-mouse CD45-APC-eFluor 780 (30-F11; Thermo Fisher Scientific), CD11b-eFluor 450 (M1/70; Thermo Fisher

Scientific), CD3-Alexa Fluor 700 (17A2; Thermo Fisher Scientific), Ly6G-APC (1A8; Thermo Fisher Scientific), MHC class II-FITC (NIMR-4; Thermo Fisher Scientific), CD11c-FITC (N418; Thermo Fisher Scientific), F4/80-PE (BM8; Thermo Fisher Scientific), B220-PE Cy7 (RA3-6B2; Thermo Fisher Scientific) and 7-AAD (Thermo Fisher Scientific) was used to exclude dead cells. Counting Beads (CountBright Absolute Counting Beads, Thermo Fisher Scientific) were used to calculate the cell number (g^{-1} kidney) as follows:

$$\text{CD45 cell absolute count (g}^{-1} \text{ kidney)} = (\text{events of CD45 cells counted} / \text{total number of beads counted} \times \text{input bead number}) / \text{g kidney.}$$

The leukocyte subset cell number (g^{-1} kidney) was multiplied by the CD45 cell number and by the percentage of the subset. For compensation, compensation beads (UltraComp eBeads; Thermo Fisher Scientific) were used. Flow cytometry data were acquired on an Attune NxT Flow Cytometer (A24860; Thermo Fisher Scientific) and analyzed by FlowJo software 10.6 (BD, Franklin Lakes, NJ, USA). The same gating strategy as previously reported was applied in this study [9].

Statistics

All data are expressed as mean \pm standard error of the mean (SEM) with individual values in dot plots. Data were analyzed using a two-way ANOVA for multiple comparisons or a Student's t-test for comparison between two groups. A P-value of $P < 0.05$ was defined as a

significant difference. All the analyses were performed with GraphPad Prism version 8.3 (GraphPad Software, San Diego, CA, USA)

Results

VNS after bilateral IRI did not protect the kidney

Dr. Inoue demonstrated that VNS after bilateral IRI attenuated the kidney injury, decreasing plasma creatinine level and the expression level of kidney Kim-1, a tubular injury marker (unpublished data). He applied VNS 1 hour, 4 hours or 24 hours after bilateral IRI and VNS 4 hours after bilateral IRI protected the kidney most. Therefore, I decided to conduct VNS 4 hours following IRI and evaluated the kidney function 24 hours after IRI. Although VNS decreased the plasma creatinine level, there was no significant difference in these groups (Figure 6). BUN was not measured in this experiment. Therefore, I moved on to another aseptic acute kidney injury model, cisplatin-induced nephropathy.

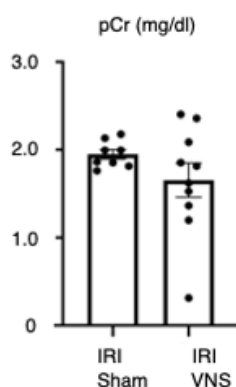


Figure 6. VNS 4 hours after IRI did not reduce the plasma creatinine level. (plasma creatinine: 1.95 ± 0.05 and 1.65 ± 0.18 mg/dl; for IRI-sham and IRI-VNS, respectively; $n = 8$ or 9). Data are expressed as mean \pm SEM and analyzed using a Student's t test. pCr, plasma creatinine; SEM, standard error of the mean

VNS 24 hours before cisplatin administration did not protect the kidney

First, I evaluated whether pre-VNS attenuated acute kidney injury in cisplatin

model as it does in IRI model. I applied VNS 24 hours before cisplatin injection and, unlike IRI model, it did not improve the plasma creatinine and BUN elevation 72 hours after cisplatin injection (Figure 7a and b). Then, I focused on the effect of VNS after the development of cisplatin-induced acute kidney injury.

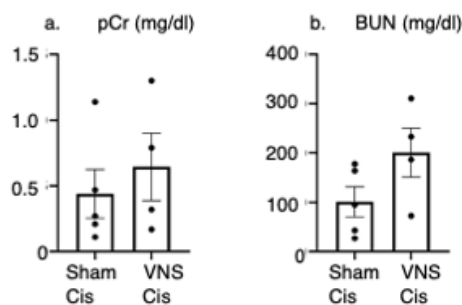


Figure 7. Pre-VNS was not effective in cisplatin-induced AKI.

(a, b) Both pCr and BUN levels were rather exacerbated by pre-VNS, though there were no statistical significance. (plasma creatinine: 0.44 ± 0.17 and 0.65 ± 0.22 mg/dl; BUN: 101.4 ± 27.4 and 200.9 ± 43.1 mg/dl; for sham-Cis and VNS-Cis, respectively; n = 4 or 5)

Cis, cisplatin

Cisplatin causes tubular damage 24 hours after its administration

Although the plasma creatinine and blood urea nitrogen (BUN) levels were not significantly elevated 24 hours after cisplatin injection (Figure 8a and b), histology demonstrated the early stage of tubular injury characterized by degenerative changes of proximal tubules (Figure 9a and b). The expression of kidney injury molecule-1 (Kim-1) and neutrophil gelatinase-associated lipocalin (Ngal) mRNA were also elevated in the cisplatin group compared to the control group (Figure 10a and b).

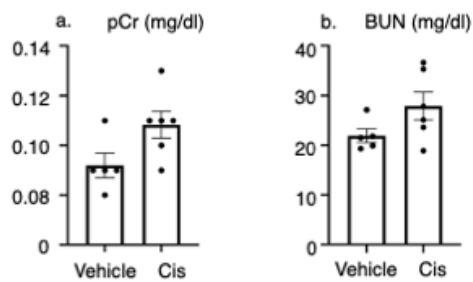


Figure 8. Kidney function parameters 24 hours after cisplatin injection.

(a, b) Plasma creatinine and BUN levels were elevated 24 hours after cisplatin injection, but the increases were not statistically significant (plasma creatinine: 0.09 ± 0.00 and 0.11 ± 0.01 mg/dl, $P=0.056$; BUN: 21.9 ± 1.2 and 27.9 ± 2.6 mg/dl, $P=0.11$; for vehicle and cisplatin group, respectively; $n=5$ or 6).

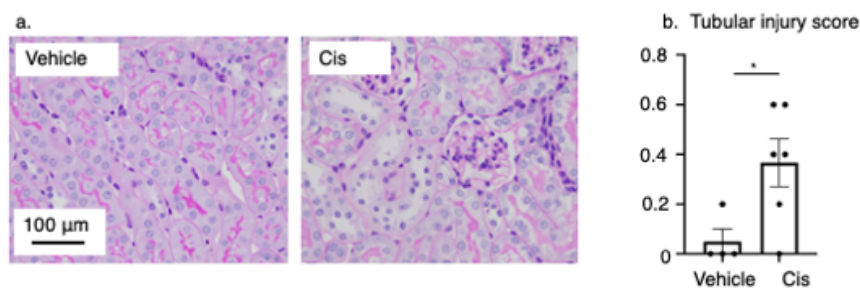


Figure 9. Kidney injury is already established 24 hours after cisplatin injection.

(a) Representative pictures of PAS staining. (b) Cisplatin caused tubular injury only 24 hours after its administration (tubular injury score: 0.05 ± 0.04 and 0.37 ± 0.10 for vehicle group and cisplatin group, respectively; $P=0.036$). $*P < 0.05$ (Student's t test), Scale bar, $100 \mu\text{m}$, PAS, periodic acid-Schiff

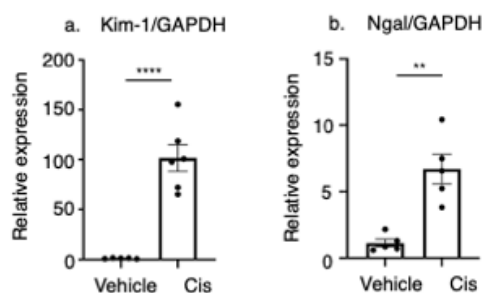


Figure 10. Kidney injury markers were also already elevated 24 hours after cisplatin

injection. (a, b) The expression levels of the early kidney injury markers Kim-1 and Ngal mRNAs in the kidney were also increased (relative expression of Kim-1: 1.10 ± 0.19 and 101.6 ± 12.2 , $P < 0.0001$; relative expression of Ngal: 1.12 ± 0.25 and 6.67 ± 1.00 , $P = 0.0013$; for vehicle group and cisplatin group, respectively; $n = 5$ or 6), $**P < 0.01$, $****P < 0.0001$ (Student's t test)

VNS after cisplatin injection protected kidney injury

Here, I applied VNS 24 hours after cisplatin injection and evaluated kidney functions in 72 hours. At 72 hours after cisplatin administration, plasma creatinine levels were increased, but decreased significantly after VNS (Figure 11a). BUN levels were not significantly different (Figure 11b).

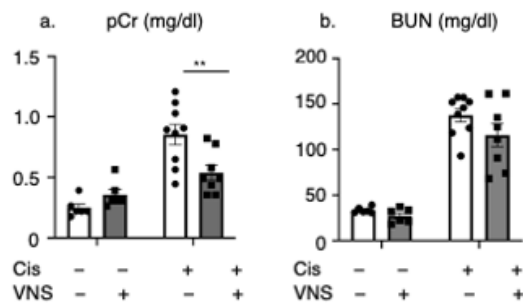


Figure 11. Effectiveness of VNS after cisplatin injection.

VNS is applied 24 hours after intraperitoneal single injection of cisplatin (25 mg/kg). (a) Plasma creatinine is significantly decreased in the VNS-treated group (plasma creatinine: 0.85 ± 0.09 and 0.53 ± 0.06 mg/dl for Cis-sham and Cis-VNS, 0.25 ± 0.03 and 0.36 ± 0.04 mg/dl for vehicle-sham and vehicle-VNS, respectively; $n = 8$ or 9 ; $P = 0.0031$). (b) BUN levels were not significant. (BUN: 137.5 ± 115.8 and 0.36 ± 12.8 mg/dl for Cis-sham and Cis-VNS, 33.4 ± 1.34 and 27.5 ± 3.17 mg/dl for vehicle-sham and vehicle-VNS, respectively; $n = 8$ or 9 ; $P = 0.13$) $**P < 0.01$, (two-way analysis of variance followed by Sidak post-hoc test.) VNS, vagus nerve stimulation

Histology showed that VNS significantly improved cisplatin-induced tubular injury, characterized by a decreased number of apoptotic or necrotic tubular epithelial cells and tubular detachments (Figure 12a and b). Increased Kim-1 expression induced by cisplatin

administration was significantly decreased by VNS treatment as seen on immunohistochemistry of the kidney (Figure 13a-c). This was further confirmed by the change in Kim-1 mRNA expression in the kidney by real-time PCR (Figure 13d).

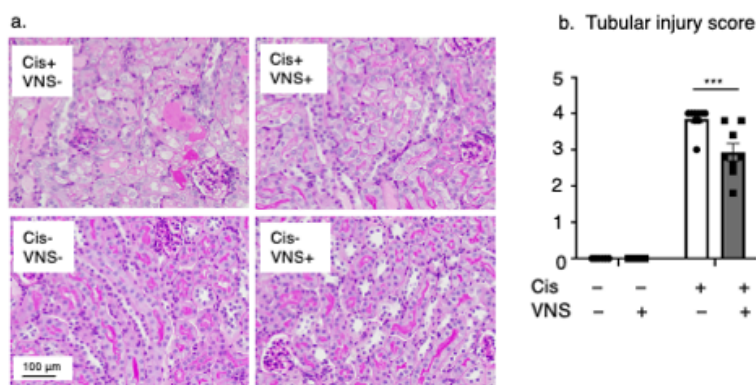


Figure 12. VNS significantly improved cisplatin-induced tubular injury.

(a) Representative pictures of PAS staining. (b) VNS attenuates the degree of tubular injury (tubular injury score: 3.84 ± 0.11 and 2.93 ± 0.25 for Cis-sham and Cis-VNS, 0.0 ± 0.0 and 0.0 ± 0.0 for vehicle-sham and vehicle-VNS, respectively; $P = 0.0002$). *** $P < 0.001$ (two-way analysis of variance followed by Sidak post-hoc test.)

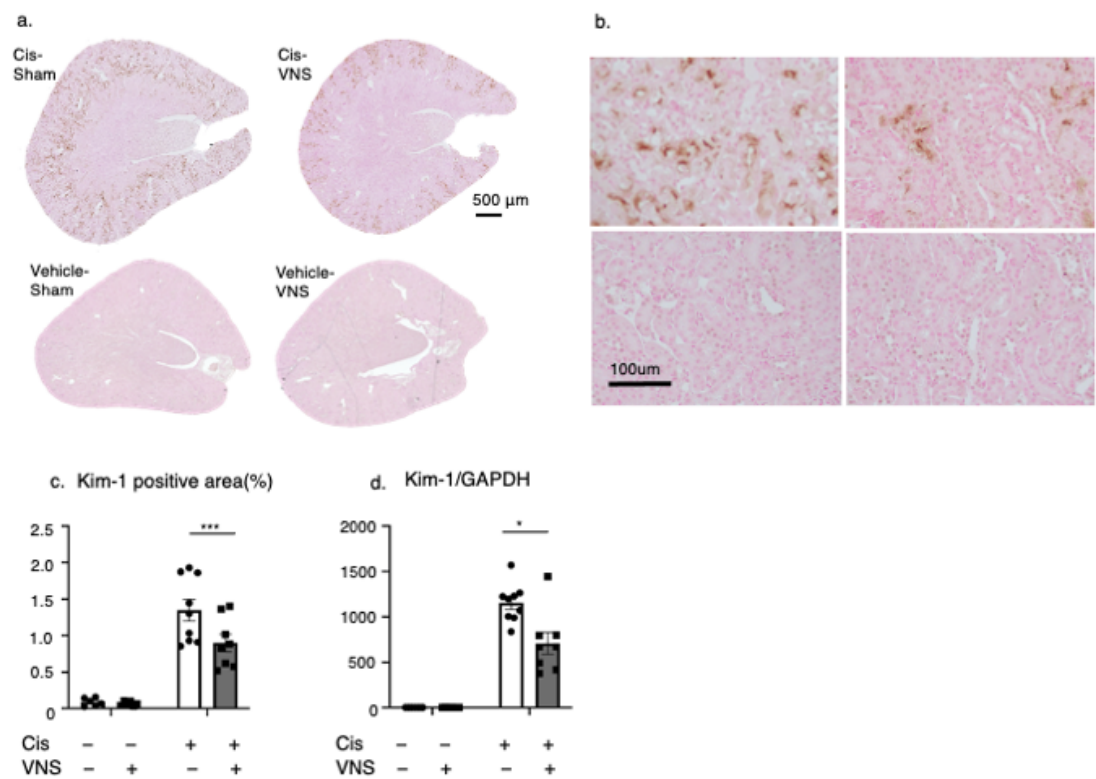


Figure 13. Increased Kim-1 expression induced by cisplatin administration was significantly decreased by VNS.

(a–d) The expression of tubular injury marker, Kim-1, is downregulated by VNS. The area fraction of the Kim-1-positive area is analyzed using Image-J software (Kim-1-positive area: 1.35 ± 0.15 and $0.90 \pm 0.12\%$ for Cis-sham and Cis-VNS, 0.09 ± 0.02 and $0.07 \pm 0.01\%$ for vehicle-sham and vehicle-VNS, respectively; $n = 8$ or 9 ; $P = 0.0133$). Kim-1 expression in the whole kidney is also reduced by VNS (Cis-sham 1154.3 ± 70.0 and Cis-VNS 708.8 ± 119.5 -fold change compared with vehicle-sham; $P = 0.0005$). Data are expressed as mean \pm SEM. Scale bar, $100 \mu\text{m}$. $*P < 0.05$, $***P < 0.001$ (two-way analysis of variance followed by Sidak post-hoc test).

Systemic administration of GTS-21 24 hours after cisplatin injection did not protect the kidney

As VNS was effective even after injury and the importance of $\alpha 7\text{nAChRs}$ on splenic macrophages was demonstrated and widely accepted [11], I evaluated the efficacy of a selective $\alpha 7\text{nAChR}$ agonist, GTS-21. Given that $\alpha 7\text{nAChRs}$ are expressed not only on splenic macrophages but also on kidney tubular epithelial cells [32] and pre-treatment with GTS-21 successfully attenuated kidney injury in LPS-induced septic AKI model [33] and cisplatin-induced AKI model [34], I expected that post-treatment with GTS-21 was renoprotective as well. On the contrary, there were no significant differences in pCr between control group and GTS groups (Figure 14). Because pCr level was not statistically different, BUN was not measured in this experiment.

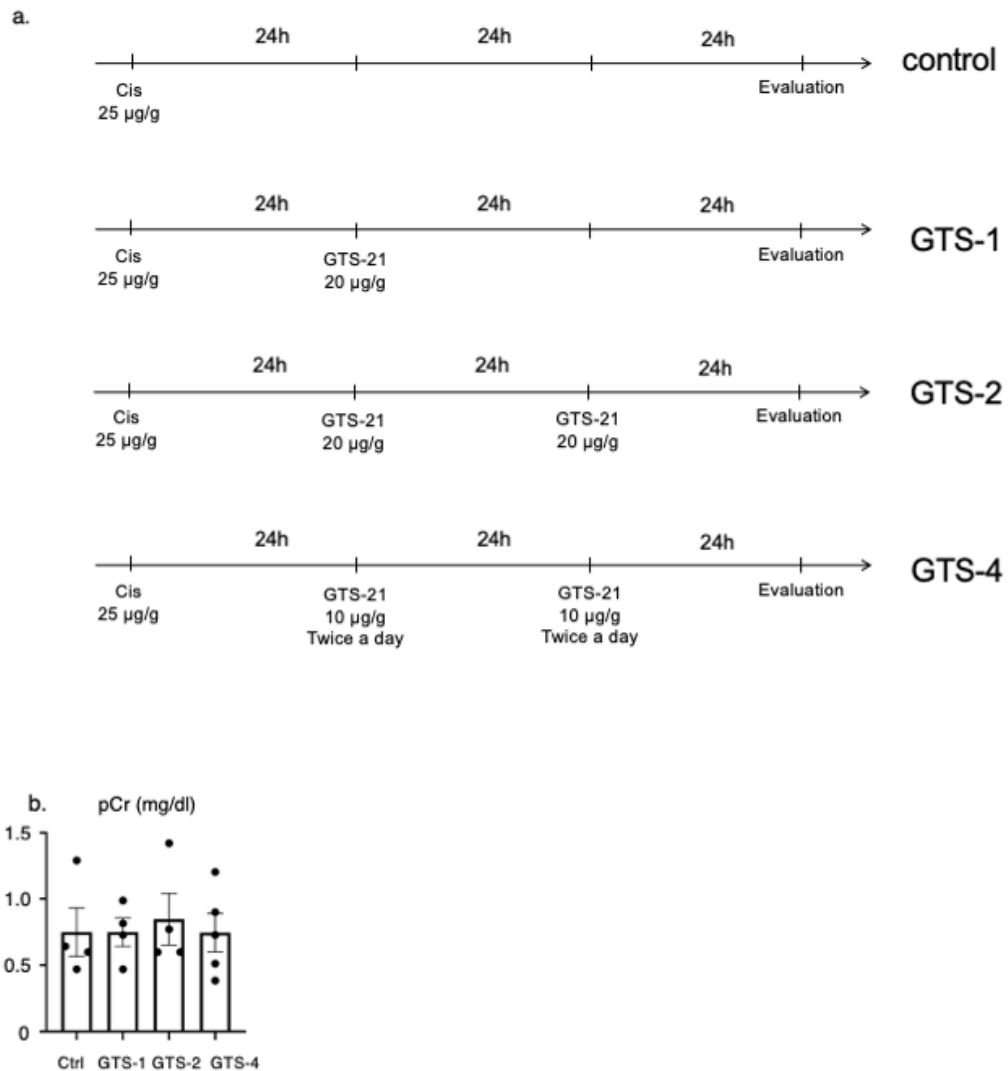


Figure 14. GTS-21 did not improve plasma creatinine levels after cisplatin administration. (a) GTS-21 administration protocol. (b) Plasma creatinine levels did not differ in these groups. (plasma creatinine: Control 0.75 ± 0.16 mg/dl, GTS-1 0.75 ± 0.09 mg/dl, GTS-2 0.85 ± 0.17 mg/dl, GTS-4 0.75 ± 0.13 mg/dl, $n = 4$ or 5)

VNS 48 hours after cisplatin injection was not renoprotective

In order to evaluate the effectiveness of VNS in a later phase, I conducted VNS 48 hours after cisplatin injection. There was no difference in pCr and BUN levels between VNS

and sham operation group (Figure 15a and b). This might indicate that 48 hours after cisplatin injection was beyond the point that VNS was effective for preventing the establishment of AKI.

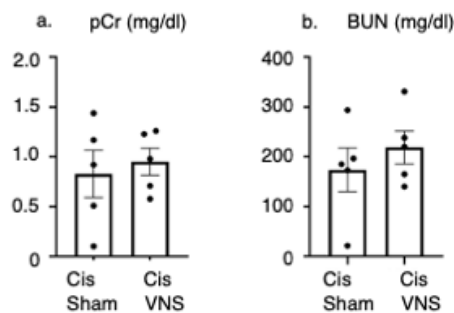


Figure 15. VNS 48 hours after cisplatin injection was not protective.

(a, b) Both plasma creatinine and BUN levels were not decreased by VNS (plasma creatinine: 1.01 ± 0.17 and 0.95 ± 0.12 mg/dl; BUN: 212.1 ± 23.9 and 218.8 ± 29.7 mg/dl; for Cis-sham and Cis-VNS, respectively; $n = 4$ or 5 , data are expressed as mean \pm SEM).

Splenectomy abolished renoprotective effect of VNS

Since the spleen is one of the essential components of the CAP and splenectomy completely eliminates the renoprotective effect of VNS in ischemia-reperfusion injury models [10], I determined to perform splenectomy experiment. First, I confirmed that splenectomy by itself did not affect the kidney function (Figure 16a and b).

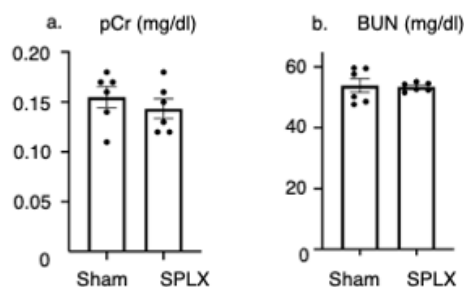


Figure 16. Splenectomy did not affect the kidney function.

(a, b) Both plasma creatinine and BUN levels were not significantly different (plasma creatinine: 0.16 ± 0.01 and 0.14 ± 0.12 mg/dl; BUN: 53.9 ± 2.29 and 53.4 ± 0.57 mg/dl; for sham and SPLX, respectively; $n = 6$, data are expressed as mean \pm SEM).

SPLX, splenectomy.

I conducted splenectomies 5 days before cisplatin injection to evaluate the contribution of the spleen to the renoprotective effect of VNS in the cisplatin-induced nephropathy model.

Renoprotection was not observed in the splenectomized mice. There was no statistical difference between the two groups in terms of plasma creatinine and BUN levels (Figure

17a and b). I also evaluated the extent of the tubular injury, which showed that VNS did not attenuate the tubular damage in splenectomized mice (Figure 18a and b).

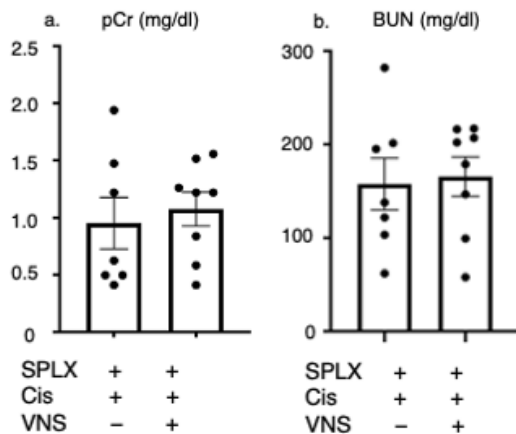


Figure 17. Splenectomy before cisplatin injection abolishes the renoprotective effect of VNS.

(a, b) Plasma creatinine and BUN do not differ between the two groups (plasma creatinine: 0.95 ± 0.22 and 1.08 ± 0.15 mg/dl; BUN: 157.7 ± 27.8 and 165.6 ± 21.0 mg/dl; for SPLX-Cis-sham and SPLX-Cis-VNS, respectively; $n = 7$ or 8).

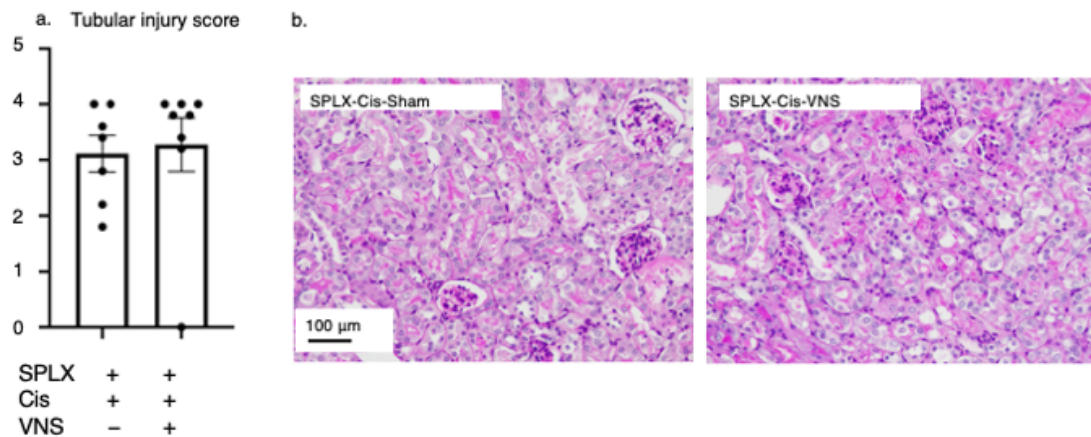


Figure 18. Splenectomy before cisplatin injection abolishes the attenuation of the tubular injury brought by VNS.

(a) Histology score do not differ between the two groups (3.11 ± 0.30 and 3.28 ± 0.49 mg/dl; for SPLX-Cis-sham and SPLX-Cis-VNS, respectively; $n=7$ or 8). (b) Representative pictures of PAS-staining. Data are expressed as mean \pm SEM and analyzed using a Student's t-test. Scale bar, 100 μ m

Adoptive transfer of GTS-21-treated macrophages improved renal outcome in the cisplatin-induced nephropathy model

Though many kinds of inflammatory cells, including B cells, T cells and dendritic cells, exist in the spleen, the anti-inflammatory effect of CAP stimulation is delivered through activation of the $\alpha 7$ nAChR on splenic macrophages [11]. I conducted adoptive transfer of splenic macrophages (1.0×10^5 cells) either treated with the selective $\alpha 7$ nAChR agonist GTS-21, or vehicle 24 hours after cisplatin injection. F4/80+ macrophages were collected using the magnetic cell separation method (MACS) and incubated with vehicle or GTS-21 for 1 hour, and then intravenously injected to recipient mice. Adoptive transfer of macrophages itself did not affect the kidney function of cisplatin-injected control mice (Figure 19), but at 72 hours after cisplatin injection, previously increased plasma creatinine

and BUN levels were significantly decreased in the mice that had received the GTS-21-treated macrophages after disease induction (Figure 20a and b). Tubular injury was also ameliorated by GTS-21-treated macrophage transfer (Figure 21a and b). The expression level of Kim-1 in the kidney was also decreased significantly in the GTS-21-treated macrophages-injected group (Figure 22).

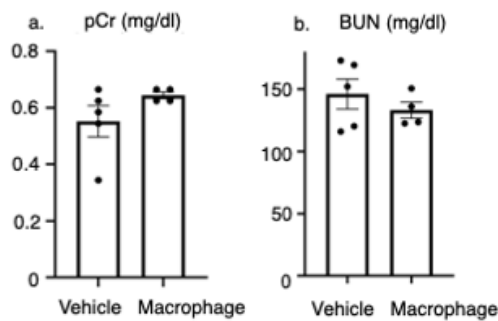


Figure 19. Adoptive transfer of macrophages did not affect the kidney function.

Macrophage group received splenic macrophages whereas vehicle group received 100 μ L of normal saline. (plasma creatinine: 0.55 ± 0.05 and 0.64 ± 0.01 mg/dl, $P = 0.0105$; BUN: 146.2 ± 12.0 and 133.2 ± 6.56 mg/dl, $P = 0.0051$; histology score: 3.33 ± 0.15 and 2.46 ± 0.26 , $P = 0.0122$; for vehicle and macrophage, respectively; $n = 4$ or 5)

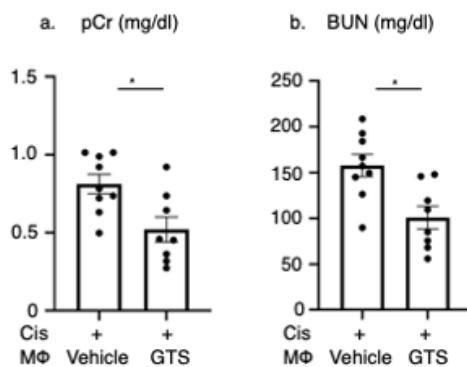


Figure 20. Adoptive transfer of $\alpha 7$ nAChR agonist GTS-21-treated macrophages improved renal outcome.

(a, b) Plasma creatinine and BUN are significantly reduced by GTS-treated macrophage transfer (plasma creatinine: 0.81 ± 0.06 and 0.51 ± 0.07 mg/dl, $P = 0.0105$; BUN: $157.8 \pm$

11.4 and 101.0 ± 11.6 mg/dl, $P = 0.0051$; histology score: 3.33 ± 0.15 and 2.46 ± 0.26 , $P = 0.0122$; for Cis-vehicle-treated macrophage and Cis-GTS-treated macrophage, respectively; $n = 8$ or 9). $*P < 0.05$ (Student's t-test) GTS, GTS-21

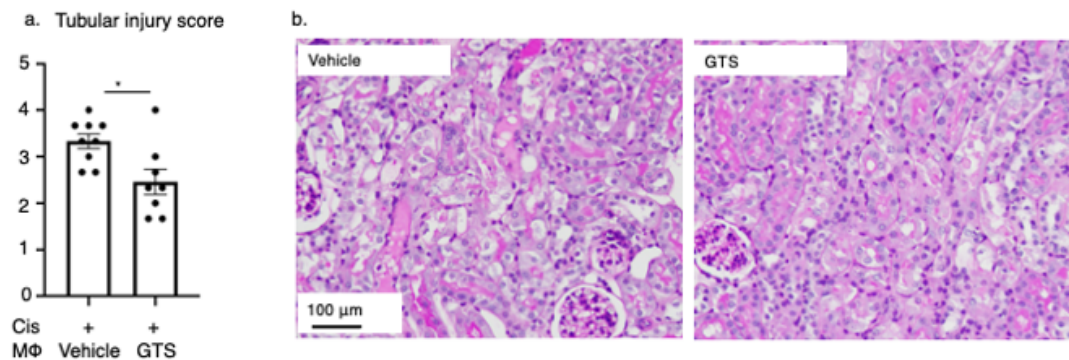


Figure 21. Tubular injury was ameliorated by GTS-21-treated macrophage transfer. (a) Histology score is significantly reduced by GTS-treated macrophage transfer (3.33 ± 0.15 and 2.46 ± 0.26 , $P = 0.0122$; for Cis-vehicle-treated macrophage and Cis-GTS-treated macrophage, respectively; $n = 8$ or 9). (b) Representative pictures of PAS-staining. $*P < 0.05$ (Student's t-test)

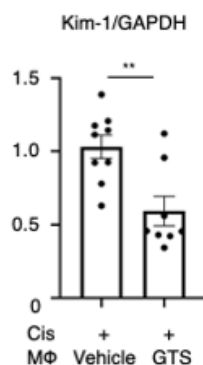


Figure 22. GTS-21-treated macrophage transfer attenuated Kim-1 elevation. The expression level of Kim-1 mRNA in the whole kidney was also decreased by GTS-21-treated macrophage transfer (relative expression of Kim-1: 1.031 ± 0.07 and 0.59 ± 0.09 , $P = 0.0034$; for Cis-vehicle-treated macrophage and Cis-GTS-treated macrophage, respectively; $n = 8$ or 9). $**P < 0.01$ (Student's t-test)

Inflammatory cytokines are upregulated in the cisplatin-induced nephropathy model and reduced by VNS

Next, in order to investigate the effect of CAP activation on systemic inflammation,

I evaluated the expression of various cytokines in plasma and created a heatmap using clustering analysis (Figure 23; raw data in Table 2).

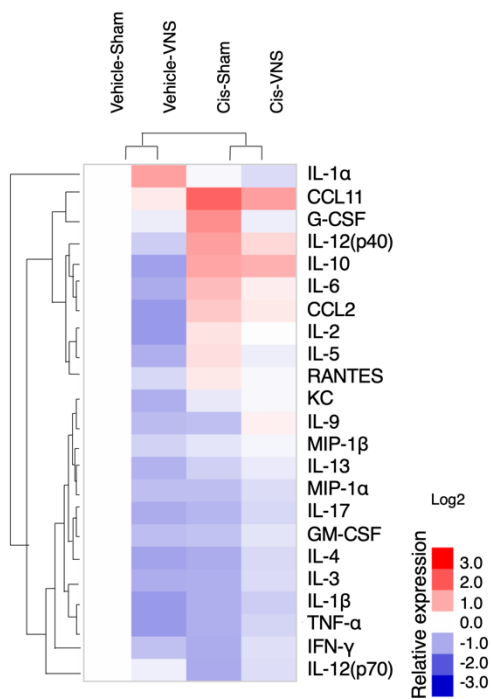


Figure 23. VNS alone downregulates the cytokines evaluated. It also suppresses most of the cytokines upregulated by cisplatin injection (raw data in Table 2). Clustering is performed to generate a heatmap using clustering software (n = 6–9). Among 23 cytokines tested, the levels of four cytokines—CCL2, CCL11 (Eotaxin), G-CSF, and IL-12(p40)—were significantly reduced by VNS in cisplatin-induced nephropathy mice (Figure 24a-d).

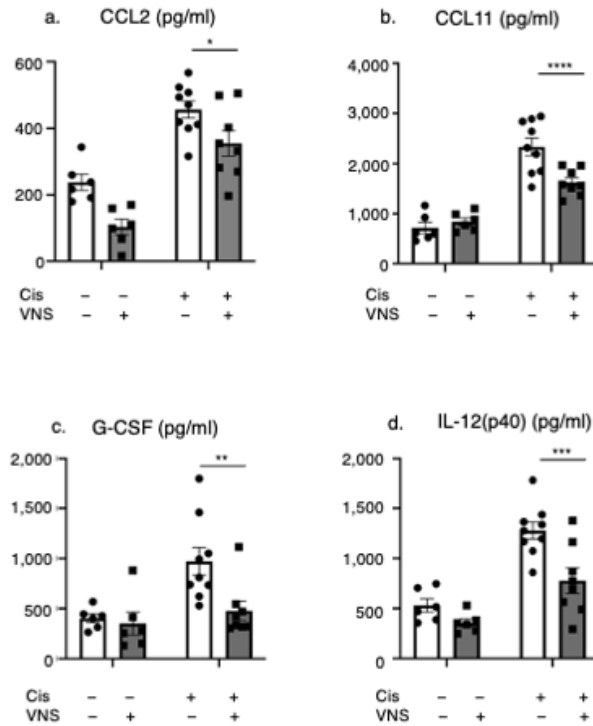


Figure 24. VNS improves systemic inflammation and decreased expression level of cytokines and chemokines in plasma. (a) CCL2 (CCL2: 368.9 ± 34.2 and 282.0 ± 36.6 pg/ml for Cis-sham and Cis-VNS, respectively; $n = 6-9$; $P = 0.0325$). (b) CCL11 (CCL11: 2565.5 ± 210.3 and 1559.1 ± 110.3 pg/ml for Cis-sham and Cis-VNS, respectively; $n = 6-9$; $P < 0.0001$). (c) G-CSF (G-CSF: 1010.4 ± 147.5 and 355.4 ± 66.4 pg/ml for Cis-sham and Cis-VNS, respectively; $n = 6-9$; $P = 0.0002$). (d) IL- 12(p40) (IL-12(p40): 1277.4 ± 85.2 and 777.2 ± 125.9 pg/ml for Cis-sham and Cis-VNS, respectively; $n = 6-9$; $P = 0.0008$). Data are expressed as mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.001$ (two-way analysis of variance followed by the Sidak post-hoc test).

Table 2

Various inflammatory cytokine expression levels in plasma (mean, pg/ml).

	Vehicle-Sham	(SD)	Vehicle-VNS	(SD)	Cis-Sham	(SD)	Cis-VNS	(SD)
IL-1 α	20.3	6.6	43.7	25.1	19.4	17.2	15.3	16.5
CCL11	715.8	247.8	843.0	167.6	2565.5	787.0	1559.1*	397.6
G-CSF	402.9	97.5	351.5	254.0	1010.4	551.9	355.4*	239.4
IL-12(p40)	529.7	148.2	355.6	90.8	1154.1	350.6	723.1*	305.8
IL-10	34.6	14.0	16.1	17.3	71.5	34.9	64.8	34.4
IL-6	13.3	5.2	6.8	5.0	23.0	10.2	15.2	10.8
CCL2	238.3	54.2	104.5	52.2	368.9	128.1	282.0*	131.9
IL-2	9.0	1.8	4.0	2.1	11.3	5.1	9.1	4.5
IL-5	19.5	6.0	10.2	6.7	25.3	13.3	17.2	7.1
RANTES	107.7	21.5	78.9	44.1	127.9	31.2	102.6	43.3
KC	202.2	33.8	106.7	51.4	169.8	46.7	191.1	54.4
IL-9	58.2	12.2	33.8	20.1	34.8	19.3	65.0	68.9
MIP-1 β	156.8	23.4	110.0	43.4	128.2	31.0	146.2	39.9
IL-13	229.1	44.9	124.3	64.9	158.2	68.9	195.4	151.5
MIP-1 α	11.6	2.1	6.9	3.4	6.9	2.6	8.8	3.3
IL-17	27.2	7.5	13.9	8.9	15.0	6.7	19.8	7.8
GM-CSF	94.3	21.2	55.6	34.2	57.8	22.4	76.4	23.9
IL-4	9.5	2.1	4.5	2.9	4.9	2.6	7.1	3.9
IL-3	6.8	2.2	3.5	1.6	3.6	1.9	5.2	2.6
IL-1 β	45.0	10.0	19.8	13.1	23.7	13.0	30.2	13.8
TNF- α	156.7	36.0	68.7	50.1	81.8	41.4	111.8	58.1
IFN- γ	95.1	18.7	57.5	33.2	48.4	30.5	73.9	31.9
IL-12(p70)	461.9	113.1	411.5	375.5	231.6	125.0	353.5	147.3

*Decreased by VNS significantly compared to Cis-sham group. (CCL11: P<0.0001, G-CSF: P=0.0002, IL-12(p40): P=0.0009, CCL2: P=0.0325) (two-way analysis of variance followed by Sidak post-hoc test)

VNS, vagus nerve stimulation; Cis, cisplatin; SD, standard deviation

Several cytokines in the kidney are elevated in the cisplatin-induced nephropathy model and downregulated by VNS

Real-time PCR showed a significant decline in the expression levels of CCL2, IL-12b, and G-CSF in the mice treated with cisplatin followed by VNS (Figure 25a-d). Since cisplatin-induced nephropathy is characterized by mitochondrial damage of tubular cells and upregulated expression of BCL2-associated X protein (BAX), which is one of the major proteins inducing mitochondrial apoptosis by permeabilizing its membrane [35,36], I evaluated the expression of BAX mRNA. As shown in Figure 25e, cisplatin-induced expression of BAX was also suppressed by VNS.

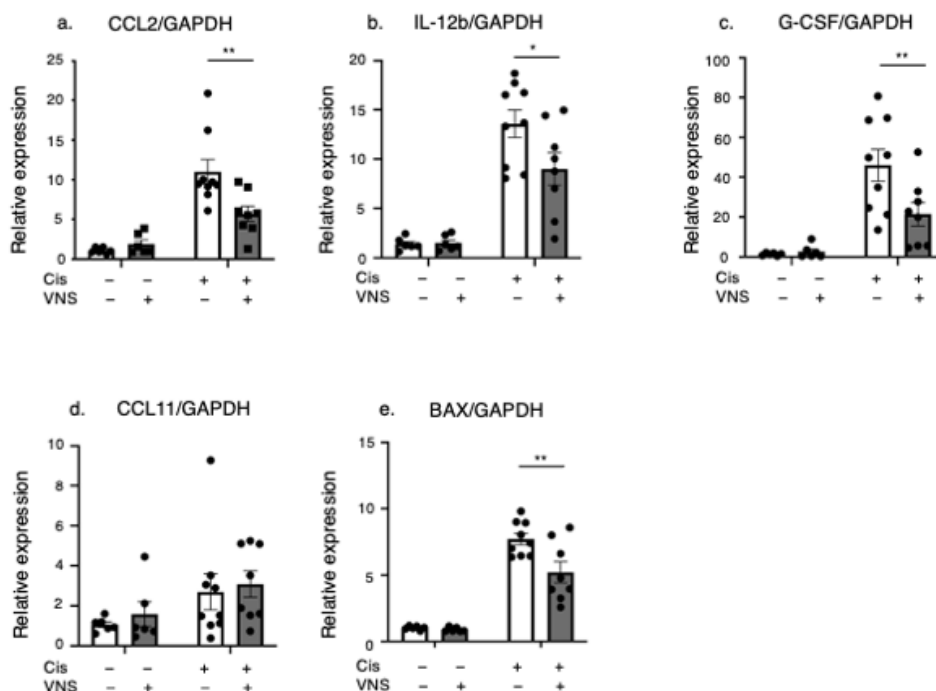


Figure 25. VNS reduces the expression of several inflammatory cytokines and chemokines in the kidney. (a) CCL2 expression (CCL2: 11.0 ± 1.53 and 5.70 ± 0.96 -fold change compared with vehicle-sham for Cis-sham and Cis-VNS, respectively; $n = 6-9$; $P = 0.0026$). (b) IL-12b expression (IL-12b: 13.6 ± 1.34 and 9.0 ± 1.66 -fold change compared with

vehicle-sham for Cis-sham and Cis-VNS, respectively; $n = 6-9$; $P = 0.0226$). (c) G-CSF expression (G-CSF: 46.0 ± 7.96 and 21.4 ± 5.86 -fold change compared with vehicle-sham for Cis-sham and Cis-VNS, respectively; $n = 6-9$; $P = 0.0089$). (d) CCL11 expression (CCL11: 13.6 ± 1.34 and 9.0 ± 1.66 -fold change compared with vehicle-sham for Cis-sham and Cis-VNS, respectively; $n = 6-9$; $P = 0.0226$). (e) BAX expression (BAX: 7.7 ± 0.43 and 5.2 ± 0.79 -fold change compared with vehicle-sham for Cis-sham and Cis-VNS, respectively; $n = 6-9$; $P = 0.0022$). Data are expressed as mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (two-way analysis of variance followed by the Sidak post-hoc test).

GTS-21 decreases CCL2 expression in macrophages

Next, as macrophages have been proven to be another key player in the CAP [11] and CCL2, one of the most potent chemokines promoting monocyte and macrophage chemotaxis, was downregulated both in the kidney and blood, I hypothesized that CAP activation prohibits macrophage migration to the injured kidneys. Therefore, I first conducted *in vitro* experiment using murine macrophage cell line RAW 264.7. These cells were divided into 4 groups receiving either cisplatin or vehicle and GTS-21 or vehicle. 24 hours later, the expression level of CCL2 was determined with real-time PCR. CCL2 was downregulated by GTS-21 in both cisplatin and vehicle treated macrophages (Figure 18).

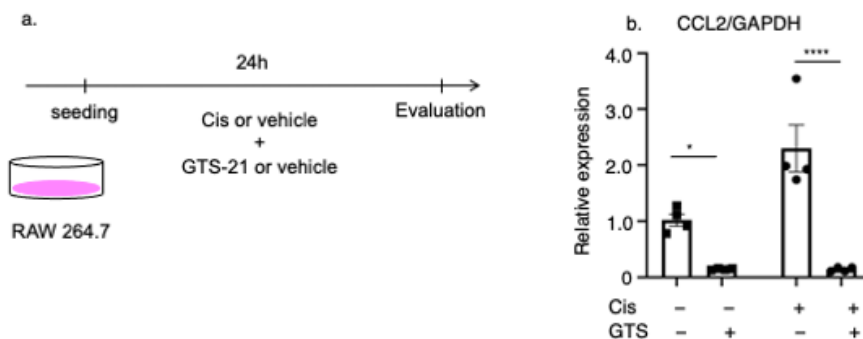


Figure 26. GTS-21 downregulated CCL2 expression in cisplatin-treated macrophages.

(a) Schematic depiction of the experimental protocol. (b) GTS-21 decreased the mRNA

expression level of CCL2 in both cisplatin- or vehicle-treated group. (0.14 ± 0.02 and 0.15 ± 0.01 -fold change compared with control for vehicle-GTS and Cis-GTS, respectively; $n = 4$; $P < 0.0001$).

Next, I performed the same experiment using human tubular epithelial cell line, HK-2 cells.

The expression of CCL2 varied in the same groups, and GTS-21 did not decrease CCL2 in cisplatin-treated group (Figure 27).

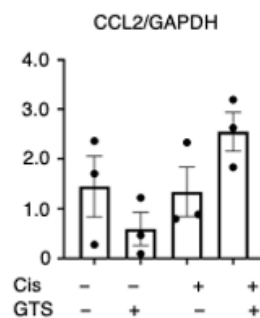


Figure 27. GTS-21 did not reduce the CCL2 expression level in cisplatin-treated human kidney tubular cells.

(0.59 ± 0.27 and 2.55 ± 0.32 -fold change compared with control for vehicle-GTS and Cis-GTS, respectively; $n = 3$)

VNS reduces F4/80+ macrophage infiltration into the injured kidney

Then, I evaluated the macrophage infiltration of the kidney in the VNS-treated or sham-treated mice after cisplatin administration by flow cytometry analysis and immunohistochemical staining of F4/80 positive macrophages. Flow cytometry analysis showed that, at 24 hours after cisplatin injection, the number of CD45-positive leukocytes, including macrophages and T cells, was slightly elevated, and the number of the cells in each leukocyte fraction was further increased 72 hours after cisplatin administration. In contrast, VNS significantly reduced the infiltration of macrophages by cisplatin (Figure 28a-e).

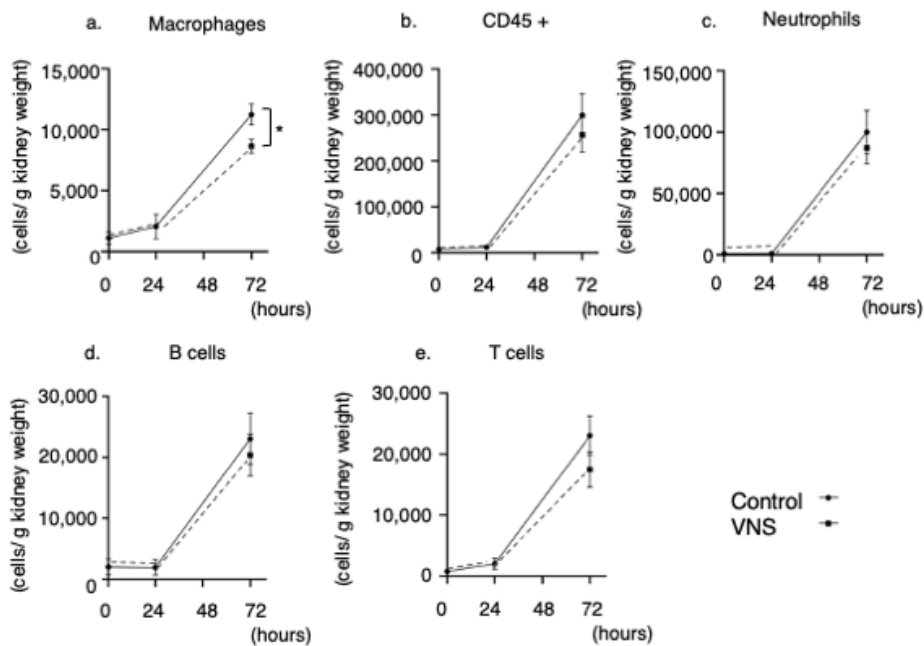


Figure 28. VNS significantly reduces the number of infiltrating macrophages in the kidney. (a–e) The number of infiltrating leukocytes is evaluated by flow cytometry analysis. (a) There is no significant difference 24 hours after cisplatin injection, but VNS significantly decreases macrophage infiltration 72 hours later (the number of infiltrating macrophages: 11261.1 ± 865.3 and 8660.8 ± 561.6 cells/ g kidney weight for Cis-sham and Cis-VNS, respectively; $n = 4$ per group; $P = 0.0453$). $*P < 0.05$ (Student's t-test)

Immunohistochemical staining of F4/80-positive macrophages also demonstrated that the number of infiltrated macrophages in the kidney was also significantly decreased by VNS at 72 hours after cisplatin injection (Figure 28a and b).

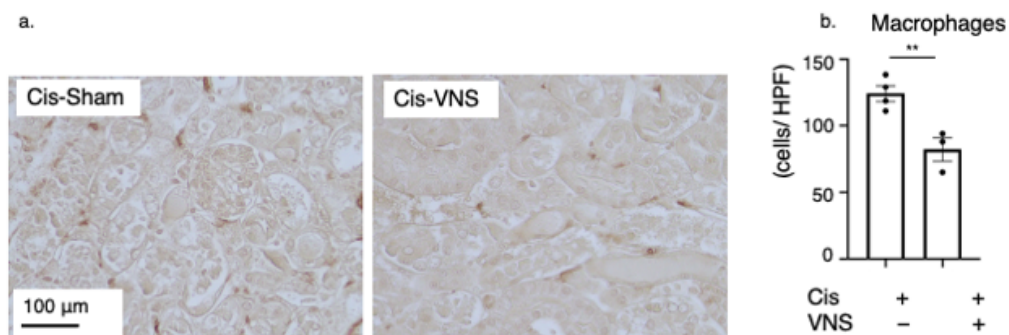


Figure 29. VNS significantly decreased the infiltration of macrophages into the kidney. (a) Representative pictures of F4/80 staining of kidney sections. (b) The number of

macrophages is counted manually using immunohistochemical staining of F4/80 positive cells. (124.3 ± 6.0 and 82.3 ± 8.8 cells/HPF for Cis-sham and Cis-VNS, respectively; $n = 3$ or 4 ; $P = 0.0095$). Data are expressed as mean \pm SEM and analyzed using a Student's t test. $*P < 0.01$.

Discussion

To the best of our knowledge, this is the first study to demonstrate the effectiveness of VNS after kidney injury.

Formerly, Dr. Inoue and Dr. Abe proved the efficacy of VNS before injury in an IRI model. Although the precise mechanism underlying the pathogenesis of AKI in toxin-induced nephropathy models and IRI models is yet to be elucidated, inflammation after initial insult is the common pathway leading to the development of kidney injury [5]. Thus, I hypothesized that VNS would be universally effective for various AKI models. More importantly, it is safe and highly feasible. Once it is approved for the treatment for AKI, this technique would benefit millions of people who develop AKI.

First, I evaluated whether VNS was effective for IRI after injury. Although VNS decreased plasma creatinine level, it was not significantly different contrary to VNS before IRI. In the previous study, pre VNS protected the kidney by promoting macrophage phenotypic change from M1 proinflammatory phenotype to M2 anti-inflammatory phenotype [10]. I speculate that in IRI model, macrophages need some time to switch their phenotypes and VNS after injury was too late (Figure 30).

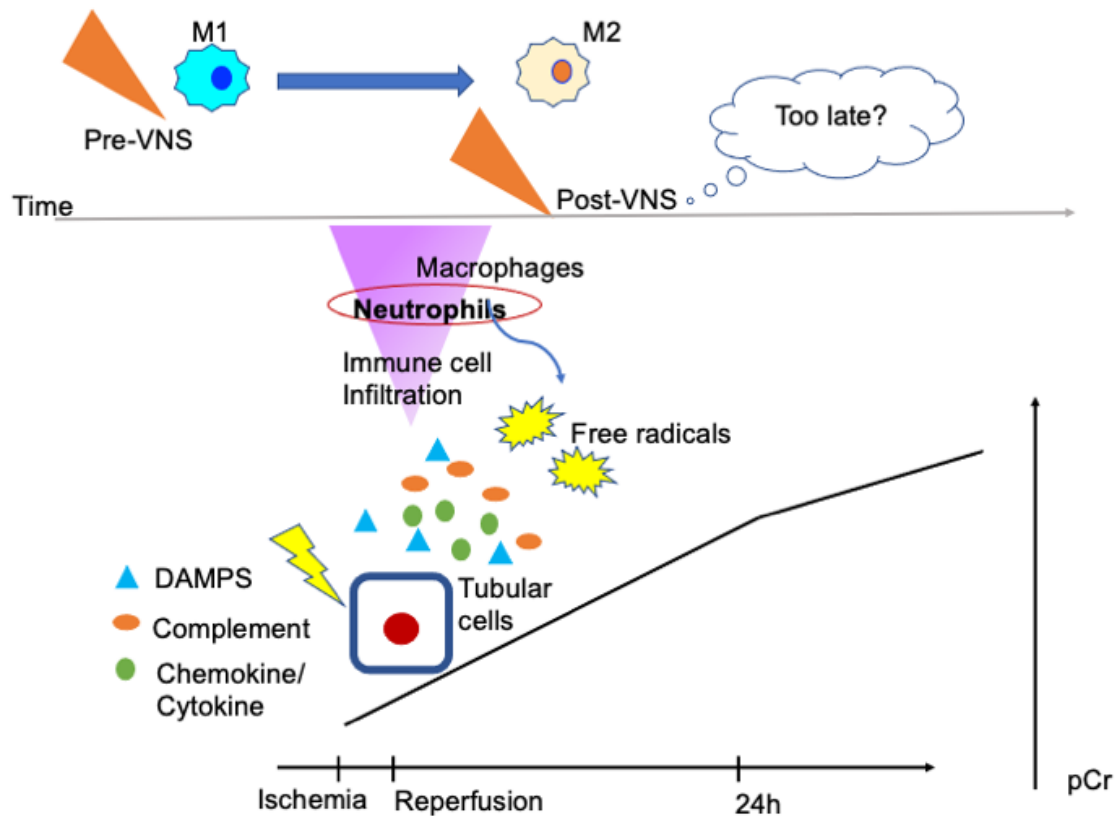


Figure 30. The relationship between IRI and VNS.

Pre-VNS promotes macrophage phenotypic change from M1 (pro-inflammatory) to M2 (anti-inflammatory). However, VNS 24 hours after IRI might be late for macrophages to change their phenotypes.

Then, I focused on another aseptic AKI model, cisplatin-induced nephropathy.

Nephrotoxicity appears in around 20–30% of patients after cisplatin administration [37]. It

is caused by the accumulation of cisplatin inside the tubular epithelial cells followed by

cellular damage, including DNA and mitochondrial damage, and ER stress [25]. In

addition to direct tubular injury, inflammation plays an important role in the pathogenesis

of cisplatin-induced nephropathy [24]. Following the initial kidney insult, renal

parenchymal cells produce several cytokines and chemokines, promoting chemotaxis of

inflammatory cells such as neutrophils, macrophages, and T cells. One to two days after

cisplatin administration, the numbers of infiltrating neutrophils and macrophages are elevated [38,39]. In this model, I first tried to show that pre-VNS exerts renoprotection, but a little surprisingly I could not see the protective effect of prior VNS (Figure 7). I presume that this result might come from the difference of kidney injury model or other factors such as the dehydration after VNS, since I observed that body weight loss was larger in VNS group compared to sham-operated mice group. Pre-existing dehydration might have made the kidney more susceptible to cisplatin-induced nephrotoxicity to the point where the damage was too severe for VNS to recover. To answer this question, further optimization for the experiment including hydration protocol would be necessary. Then, I applied VNS 24 hours after cisplatin injection, when early tubular injury was already established, and it successfully attenuated the acute kidney injury (Figures 11-13). VNS 48 hours after drug administration was not effective (Figure 15). This might indicate that 48 hours after cisplatin injection was beyond the point that VNS was effective for preventing the establishment of AKI. Considering the timing of VNS application, I inferred that VNS after injury, in contrast to VNS before injury, exerts a renoprotective effect by inhibiting further leukocyte infiltration into the kidney (Figure 31).

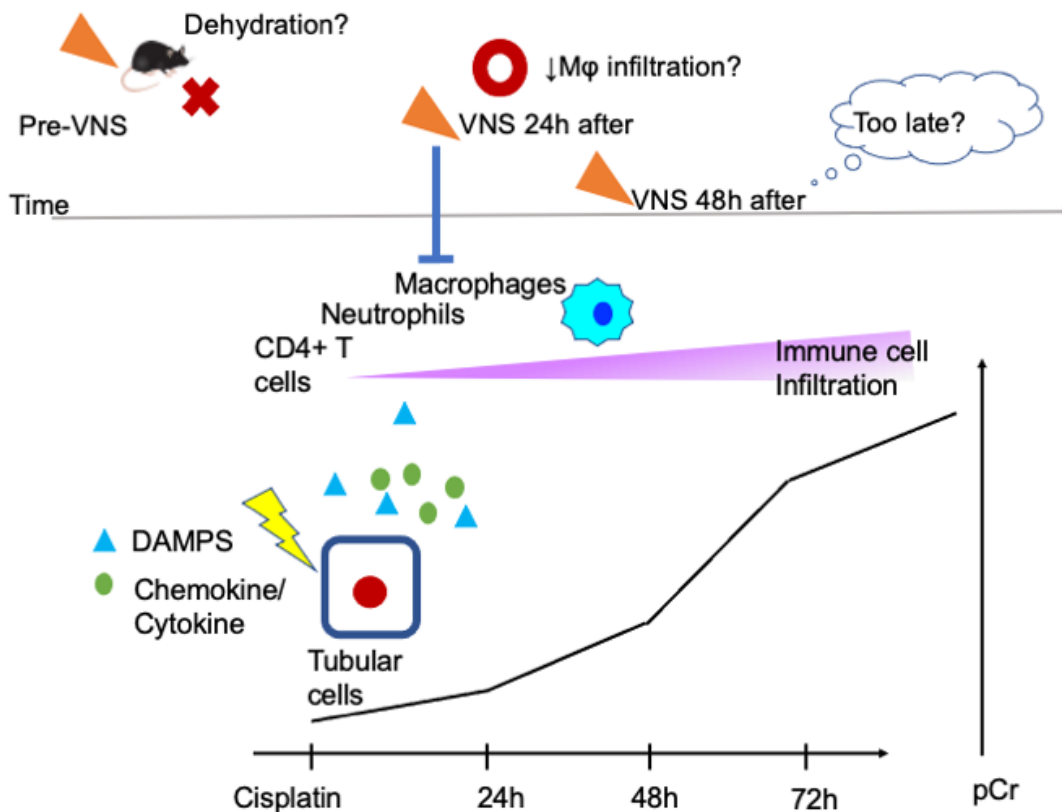


Figure 31. VNS and cisplatin-induced nephropathy.

Pre-VNS was not effective possibly because pre-existing dehydration caused by VNS made cisplatin-induced tubular damage severer. VNS 24 hours after cisplatin administration protected the kidney by reducing the number of infiltrating macrophages, while VNS 48 hours was not effective.

Mφ, macrophage

Here, I will briefly review the CAP to focus on the precise renoprotective mechanism underlying VNS. CAP is composed of both afferent and efferent arms and both afferent and efferent vagus nerves play important roles. Although its precise mechanism is still to be elucidated, it is estimated that the efferent vagus nerve synapses with the splenic nerve [40]. The activated splenic nerve stimulates CD4⁺ CD25⁻ T cells (non-regulatory T cells) in the spleen via activation of β 2-adrenanergic receptors on those cells [41]. A subset of CD4⁺ T cells (CD4⁺CD44^{high} CD62L^{low} memory T cells) in the spleen possess the

acetylcholine biosynthetic enzyme, choline acetyltransferase (ChAT), and can synthesize acetylcholine [42], and subsequently, those T cells are supposed to interact with $\alpha 7$ nicotinic acetylcholine receptors on splenic or peritoneal macrophages [43]. The production of pro-inflammatory cytokines, such as TNF- α and interleukins, is suppressed in these activated macrophages. Dr. Inoue, for the first time, documented the importance of the peritoneal macrophages in activation of the CAP in vivo [43]. Even though the exact relationship between each type of immune cell is not fully understood, the spleen serves as an important place for immune cell interaction and a reserve for immune cells.

Therefore, I conducted splenectomy experiment to show the importance of the spleen in renoprotective effect of VNS in cisplatin induced AKI. And renoprotection was completely abolished by splenectomy similar to the IRI-induced tubular injury (Figure 17, 18) [10]. This is consistent with the previous report demonstrating the importance of the spleen in the CAP [44].

Adoptive transfer of GTS-21-treated macrophages further reinforced the importance of macrophages in the CAP (Figure 20-22). As it has been proved that both splenic and peritoneal macrophages were essential in CAP activation [10,43], this systemic administration of stimulated macrophages mimics a situation where either splenic or peritoneal macrophages interact with other cells in various organs. The number of transferred macrophages was relatively small, but their anti-inflammatory effect was potent

enough to suppress kidney dysfunction and tubular injury. Thus, I speculate that these cells interact with other immune cells and hinder the proinflammatory process (Figure 32).

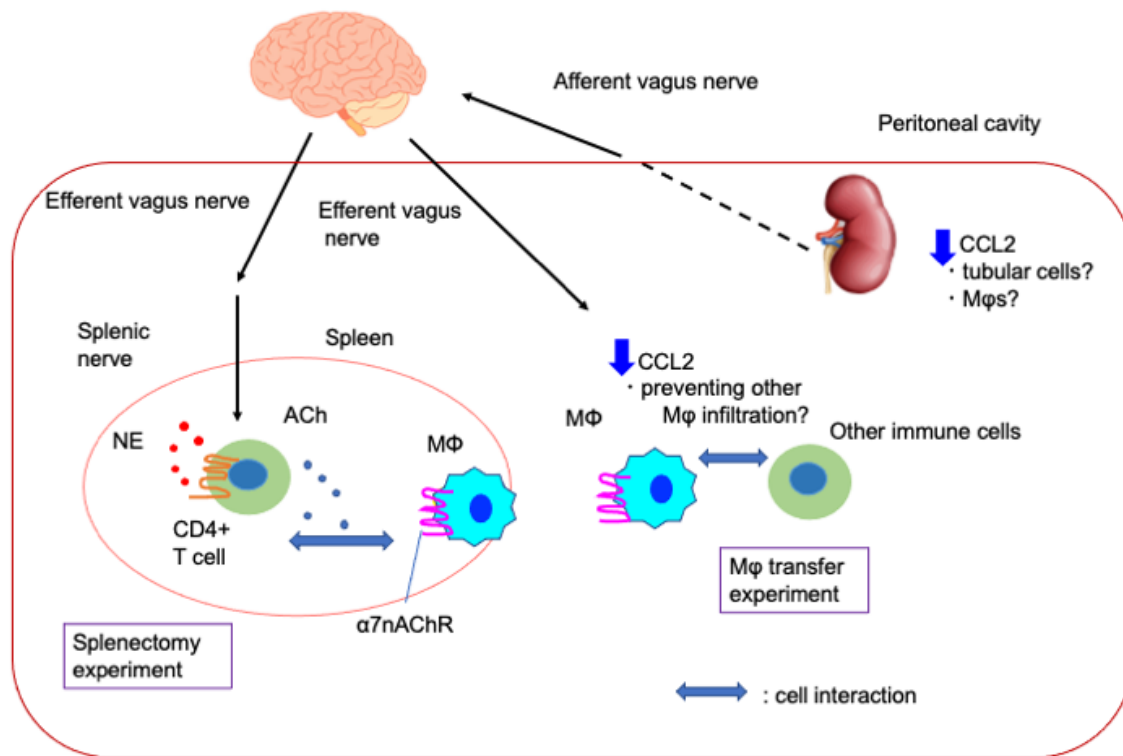


Figure 32. Summary of the CAP and experiments.

Splenectomy experiment displayed the importance of the spleen in the CAP serving as a reservoir and the place for immune cell interaction. Macrophage transfer experiment demonstrated that the interaction between macrophages and other immune cells or cells in tissues was necessary for anti-inflammatory effect of the CAP. CAP activation reduces CCL2 and other cytokine release either from circulatory macrophages, tissue resident macrophages or kidney cells and prevents macrophage infiltration into the injured kidney. Mφ, macrophage

Multiple cytokine assay revealed that VNS significantly decreased several chemokines and cytokines. Cisplatin-induced nephropathy is characterized by elevation of pro-inflammatory cytokines such as TNF- α , IL-1 β , IFN- γ , GM-CSF, MIP-1, KC, IL-18, IL-6, IL-12 and MCP-1 (CCL2) and anti-inflammatory cytokine IL-10 [23,24,45]. For

some reason, except for the latter four cytokines, cytokines that were previously reported to be elevated in cisplatin-induced nephropathy were not elevated in my experiment. This discrepancy might arise from the difference of the samples, because most of the studies measured kidney tissue cytokine expression levels, not plasma levels, or from the difference of the breeding environment. Yet, the fact that cytokines related to leukocyte infiltration were significantly reduced by VNS supported my hypothesis. In IRI or unilateral ureteral obstruction (UUO) mice, G-CSF is upregulated and the number of neutrophils increases in the injured kidney [46]. IL-12 and IL-33 work together as a stimulant to invariant natural killer T cells in the kidney and promote neutrophils and monocytes/macrophage infiltration into the organ [47]. Another study also reported that IL-12 deficient mice had better renal outcomes in an IRI-induced AKI model [48].

CCL2 (MCP-1) is one of the most potent chemokines, attracting monocytes and macrophages [49]. Circulating CCL2 first recruits monocytes from the bone marrow, and those monocytes are recruited by locally produced CCL2 into the inflamed organs. Following this, local CCL2 induces differentiation and cytokine production of monocytes/macrophages in the kidney. Its pathogenic role has been reported in various kidney diseases, such as membranous nephropathy, SLE, diabetic nephropathy, and autosomal polycystic kidney disease (ADPKD) [49,50].

Among these cytokines and chemokines, which were suppressed in both plasma

and the kidney (Figure 23, 24), I focused on CCL2, since it recruits macrophages, one of the main role players in the CAP. Knocking out CCL2 in the renal tubular cells decreases macrophage infiltration into the kidney and ameliorates cyst formation in ADPKD [50]. In a diabetic nephropathy model, blockade of CCL2/CCR2 signaling using a CCR2 antagonist significantly reduced renal macrophage infiltration and attenuated histological changes caused by diabetes [51]. The pathogenic role of macrophages in AKI has been well established in an IRI mouse model [52]. In the first 48 hours after injury, inducible NO synthase (iNOS)-expressing pro-inflammatory M1 phenotype macrophages predominate in the kidney, whereas in the later phase, arginase-1 (Arg-1)-positive anti-inflammatory macrophages dominate. In cisplatin-induced nephropathy mouse models, reduction of macrophage infiltration, and attenuated pathological changes, and kidney dysfunction are correlated [53,54].

In the field of rheumatoid arthritis, the relationship between activation of the CAP and attenuation of arthritis via suppression of macrophage infiltration has been reported [55]. GTS-21, a selective agonist for the $\alpha 7$ nAChR, effectively decreased the expression level of CCL2 in cisplatin-treated macrophages (Figure 26). I tried to show that GTS-21 also decreases the CCL2 from tubular epithelial cells using HK-2 cells, but the wide variety of expression levels needs further optimization. Although various renal cell types including mesangial cells, glomerular cells, endothelial cells, macrophages and CD4⁺ T cells are the

source of cytokines in the kidney [56,57], reduced CCL2 expression in GTS-treated macrophages supports our in vivo data that showed that VNS reduces macrophage infiltration into the kidney via suppression of CCL2 (Figure 25a, 27a). It is a little complicated, but macrophages do both good and harm. Once they are stimulated by CAP activation, they either change their phenotype from pro-inflammatory M1 to anti-inflammatory M2 or decrease the release of inflammatory cytokines prohibiting further macrophage infiltration. Without the stimulation, macrophages infiltrate in the injured kidney and accelerate the inflammation. Taken together, these findings suggest that VNS inhibits the pro-inflammatory macrophages into the kidney and ameliorates tubular injury and kidney dysfunction.

In summary, this study documents for the first time the link between macrophage infiltration in the kidney and CAP stimulation, possibly through the modulation of CCL2 expression (Figure 23-27). Activation of the CAP inhibits NF- κ b [58] and the JAK2/STAT3 [59] pathway, which are major transcriptional pathways for CCL2. In IRI models, VNS pretreatment did not affect the number of macrophages in the kidney [10], and this discrepancy arises from differences in kidney injury models or differences in the timing of VNS application. Future studies will be needed to clarify the complex molecular mechanisms underlying the relationship between CAP activation and macrophage migration. Clinically, AKI is one of the most common complications in critically ill patients. Although

there are treatment options to support patients' kidney functions including intermittent hemodialysis (IHD) or continuous renal replacement therapy (CRRT), they are invasive and sometimes cause treatment-related complications such as hemorrhage and infection. Some patients do not fully recover and become dependent on renal replacement therapy (RRT) for the rest of their lives. In Japan, the mainstream of RRT is hemodialysis, where patients have to go to clinics on a regular basis basically 3 times a week, and each session lasts for 4-5 hours. This is a big burden for patients both physically and mentally. If VNS could prevent the development of severe AKI and decrease the number of patients who newly started dialysis, it would be very beneficial. Here, I only demonstrated that VNS was effective for cisplatin-induced acute kidney injury. But if VNS is applicable and effective for more chronic kidney diseases such as diabetic kidney disease (DKD), hypertensive nephropathy, and nephritis, it would definitely become a breakthrough for nephrological treatment. Especially, current treatment options for nephritis are immunosuppressants or plasma exchanges, and the former is not tolerable for some patients due to side effects and the latter is more or less invasive. Therefore, safe and less invasive VNS would be a promising treatment tool. In addition, it is advantageous not only for patients, but also for national medical expenditure, as hemodialysis is an expensive treatment costing around 400,000 yen a month per patient. In order to utilize this innovative technique, future studies are needed to show the effectiveness of VNS for other types of kidney disease both acute and chronic.

In conclusion, I have demonstrated for the first time that VNS after injury protects the kidney from cisplatin-induced nephropathy via CAP activation and prohibition of inflammatory cell infiltration into the kidney. VNS is safe and has potential as a potent therapeutic tool for cisplatin-induced nephropathy.

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