CD4+ CD25+ regulatory T cells partially mediate the beneficial effects of FTY720, a sphingosine-1-phosphate analogue, during ischaemia/reperfusion-induced acute kidney injury

Myung-Gyu Kim1, So Young Lee2, Yoon Sook Ko1, Hee Young Lee1, Sang-Kyung Jo1, Won Yong Cho1 and Hyoung-Kyu Kim1

1Division of Nephrology, Department of Internal Medicine, Korea University Anam Hospital, Korea University College of Medicine, Seoul, Korea and 2Department of Internal Medicine, Eulji General Hospital, Eulji University College of Medicine, Seoul, Korea

Correspondence and offprint requests to: Sang-Kyung Jo; E-mail: sang-kyung@korea.ac.kr

Abstract

Background. The synthetic sphingosine-1-phosphate (S1P) analogue, FTY720, attenuates ischaemia/reperfusion (I/R) injury by inducing peripheral lymphopaenia. Recent studies suggest that FTY720 may also exert protective effects by modulating dendritic cell (DC) function or directly affecting regulatory T cells (Tregs). The purpose of the present study was to examine whether the beneficial effect of FTY720 in I/R-induced acute kidney injury (AKI) involves modulation of DCs or Tregs.

Methods. Mice underwent bilateral ischaemia, and FTY720 or vehicle was then administered. Biochemical values, histological kidney damage and tissue inflammation were assessed. Phenotype and function of DCs in blood/spleen or kidney were also examined by flow cytometry or mixed lymphocyte reaction (MLR) assay. Percent Tregs or FoxP3 mRNA expression was examined in kidney and spleen, and depletion and adoptive transfer of Tregs were also performed.

Results. Treatment with FTY720 attenuated I/R kidney injury and reduced inflammation. The beneficial effect of FTY720 was associated with expansion of peripheral CD11b+ CD11c+ DC and with maturation of spleen CD11c+ DC, which showed impaired allostimulatory capacity. FTY720-treated animals also showed a higher frequency of CD4+ CD25+ Tregs and an upregulation of FoxP3 mRNA expression in spleen and kidney. In vitro experiments showed that FTY720 induced expansion of Tregs, possibly via conversion from non-Tregs to Tregs. Depletion and adoptive transfer of Tregs were associated with loss and recovery of the beneficial effects of FTY720.

Conclusion. These results suggest that the beneficial effects of FTY720 in I/R injury may be partially mediated by DC modulation or by increasing Treg activity. Further studies that identify tolerance induction mechanisms will be useful for developing strategies for the prevention or treatment of AKI.

Keywords: acute kidney injury; dendritic cell; FTY720; regulatory T lymphocyte

Introduction

A growing body of evidence supports the concept that inflammation contributes to acute kidney injury (AKI). Numerous compounds with anti-inflammatory effects have been tested for their ability to attenuate kidney injury in animal models of ischaemia/reperfusion (I/R)-induced AKI.

A synthetic sphingosine-1-phosphate (S1P) analogue, FTY720, has been used in autoimmune and allergic diseases as well as in solid organ transplantation because of its known immunosuppressive function. Beneficial effects of the compound have been ascribed to its ability to induce sequestration of lymphocytes into secondary lymphoid organs, causing subsequent depletion of lymphocytes in blood to further prevent migration of these cells towards the site of inflammation.

Recently, FTY720 was also found to be effective in reducing I/R-induced kidney injury through its ability to induce peripheral lymphopaenia [1,2]. However, FTY720 may have effects that are independent of lymphopaenia induction since VPC-44116, which is a selective S1P1 receptor antagonist that blocks FTY720, did not cause a reversal of lymphopaenia. In addition, Bajwa et al. [3] recently demonstrated that FTY720 or an S1P1 agonist exerted renoprotective effects via tubular cell S1P1-S1P1 signalling effects that were independent of peripheral lymphopaenia induction. Furthermore, several other studies suggested that the anti-inflammatory effects of FTY720 may be due to its tolerogenic activity by modulating dendritic cell (DC) function or by a direct effect to increase CD4+ CD25+ regulatory T cell (Treg) activity [4–9]. Tregs are known to play a critical role in the main-
tenance of immunologic tolerance, preventing transplant rejection, and in controlling inflammation in various disease models. According to two recent reports, Tregs are thought to contribute to the suppression of innate immune responses or a facilitation of the recovery process following kidney I/R injury [10,11]. These findings raise the possibility that the beneficial effects of FTY720 may be mediated not only by peripheral lymphopaenia but also by its effect on DC function or on Tregs. In a Th1-mediated model of colitis, FTY720 induced anti-inflammatory effects that were associated with increased FoxP3 expression in isolated CD4+ T cells in Payer’s patch and colon tissues [8]. FTY720 was also found to be effective in viral-induced immunopathology by inducing conversion of conventional FoxP3- T cells to FoxP3+ regulators [9]. The purpose of the current study was to investigate whether beneficial effects of FTY720 in I/R-induced AKI are partially mediated by a modulatory effect on DCs or by an effect on expanding Tregs population.

Materials and methods

Animal experiments

Six-to-eight-week-old male C57BL/6 mice (weight, 20–25 g) were purchased from the Orient (Charles River Korea, Seoul, Korea) and were given free access to water and chow before the experiment. Animal care followed the criteria of the Animal Care Committee of Korea University for the care and the use of laboratory animals in research. The mice were subjected to bilateral renal pedicle clamping for 32 min, and sham operations were performed using a similar surgical procedure except for the clamping of the renal pedicles. At 24 h after I/R, the animals were sacrificed, blood was collected by intracardiac puncture, the spleen and both kidneys were perfused with PBS and then processed for molecular and histologic examinations. For FTY720 treatment, FTY720 (240 μg/kg) or the same volume of vehicle was administered through intraperitoneal injections three times at 48 and 24 h before ischaemia, and at 30 min after ischaemia.

Biochemical analysis

Blood (400 μL) was collected at 24 h after I/R, and serum creatinine was measured using a Hitachi 747 automatic analyser.

Histological examination

Tubular injury was semiquantitatively assessed in periodic acid Schiff (PAS) stained kidney tissues and also by TUNEL staining. For immunohistochemical detection of monocytes/macrophages or neutrophils, we stained formalin-fixed and paraffin-embedded kidney sections with monoclonal antibody against F4/80 (Serotec, UK) or Naphthol AS-D chloroacetate (Sigma-Aldrich). Eight to ten high-power fields (HPFs) were captured, and the mean number of F4/80 or esterase positive cells per HPF was compared.

**Fig. 1.** Effect of FTY720 on peripheral CD4+, CD8+ cells, renal function and histology after I/R injury. (A) FTY720 treatment induced marked peripheral lymphopaenia (n = 4 per group). (B) FTY720-induced peripheral lymphopaenia was associated with decreased serum creatinine levels, tubular damage and tubular cell apoptosis (n = 7–8 per group). *P < 0.05 compared to sham, **P < 0.05 compared to vehicle + I/R. PAS, TUNEL, ×100.
Quantification of cytokines and chemokine by cytometric bead array

Quantification of various cytokines and chemokine in kidney tissues was done using cytometric bead array (CBA). A mouse inflammation kit (BD Bioscience, San Diego, CA, USA) was used according to manufacturer’s instructions to simultaneously detect mouse IL-12p70, TNF-α, IFN-γ, MCP-1, IL-10 and IL-6, as previously described. Briefly, a mixture of six capture bead populations (50 μL) with distinct fluorescence intensities (detected in FL3) coated with antibodies specific for the above cytokines and chemokine was mixed with each sample from kidney homogenates/standard (50 μL/tube). Standard dilutions and test samples were added to the appropriate sample tubes (50 μL/tube). Additionally, PE-conjugated detection antibodies (detected in FL-2; 50 μL) were added to form sandwich complexes. After 2 h of incubation at room temperature, the samples were washed with 1 mL of wash buffer and centrifuged (200 g for 5 min). Three hundred microlitres of wash buffer was added to each assay tube before acquisition in a FACScan flow cytometer (FACSCalibur™, BD Biosciences), and the sample results were analysed using CBA software (BD Biosciences). The concentration for each cytokine in cell supernatants was determined by interpolation from the corresponding standard curve and normalized according to protein concentrations. The range of detection was 20–5000 pg/mL for each cytokine, and kidney cytokine levels were normalized to milligramme protein.

Determination of spleen and kidney CD11c+ DC phenotype

To determine the effect of FTY720 on DC phenotype, mononuclear cells (MNCs) from blood, spleen and kidney were isolated. For kidney MNC isolation, both perfused kidneys were minced and digested in collagenase type I (Sigma-Aldrich) at 37°C for 30 min. The collagenase-digested tissue was gently pressed through a 100-μm cell strainer and then washed with 40 mL of PBS. The pellet, suspended in HBSS, was gently overlaid onto Percoll and centrifuged at 900 g for 20 min. Isolated cells from the Percoll interface were washed with PBS and then used for kidney MNC analysis. These cells were stained for 15 min on ice with fluorochrome-labelled monoclonal antibodies (CD45, CD11c, CD11b, CD80, CD86 and MHC II) (eBioscience or Miltenyi Biotec) and were analysed using four-colour flow cytometry (FACSCalibur™, BD Bioscience). To further determine the function of DCs from FTY720-treated mice, a one-way MLR was performed. Stimulator cells were prepared from spleens of vehicle + I/R or FTY720 + I/R mice by treating them with mitomycin C (50 μg/mL, Sigma-Aldrich, MO, USA) for 15 min at room temperature, followed by five washes and then incubation with responder T cells from BALB/c mice (pan T cell isolation kit, Miltenyi Biotec, USA). Responder (1 × 10⁶) and stimulator cells (4 × 10⁵, 2 × 10⁶) were added to round bottom 96-well plates to a final volume of 200 μL of RPMI 1640 and were incubated for
72 h. Then, BrdU was added for the final 18 h of culture, and the degree of T cell proliferation was compared by measuring bromodeoxyuridine (BrdU) incorporation compared to stimulator cell only plates.

**Flow cytometric analysis of Tregs**

To determine the effect of FTY720 on Treg population, flow cytometric detection of CD4+CD25+ or CD4+FoxP3+ Tregs in kidney and spleen was performed. In addition, in vitro experiments assessing the effect of FTY720 on the induction of Tregs from naïve T cells were also performed. One hundred thousand splenocytes (10^5) were placed in 96-well plates, and FTY720 (10 ng/mL) or vehicle was added daily with IL-2 (25 U/mL). On day 4 after drug treatment, cells and supernatants were harvested for flow cytometric detection of CD4+FoxP3+ Tregs, FoxP3 mRNA expression and cytokine concentrations that included TGF-β. In a separate experiment, anti-TGF-β monoclonal Ab was coincubated, and percent Tregs was measured by flow cytometry. BrdU was also added to each well for the last 18 h, and its uptake was compared between the two groups (FTY720 vs vehicle-treated cells).

**In vivo depletion of Tregs and adoptive transfer experiments**

For depletion of Tregs, rat anti-mouse CD25 mAbs (PC61), which was obtained from ascitic fluid produced by PC61 hybridoma (ATCC, MD, USA) in nu/nu mice (Charles River Korea, Seoul, Korea) and purified by Prosep G Ig purification kit (Millipore, Bedford, MA), was administered intraperitoneally to C57BL/6 mice at a dose of 0.3 mg/mice with FTY720 at 5 days before ischaemia. As a negative control, rat isotype-control antibody was used at the same dose. Depletion of CD4+CD25+ cells was confirmed by flow cytometric examination of spleen. For adoptive transfer experiments, CD4+CD25+ cells were isolated from spleen by using a MACS Treg isolation kit (Miltenyi Biotec, Germany), and 1 × 10^6 cells were adoptively transferred into Treg-depleted mice.

**Fig. 2.** Effect of FTY720 on inflammation after I/R injury. (A) Tissue cytokines measured by cytometric bead array showed decreased levels of the pro-inflammatory cytokine, IL-6, but increased tissue levels of the anti-inflammatory cytokine, IL-10, in FTY720-treated mice. (B–D) FTY720 treatment resulted in a reduced infiltration of F4/80+ macrophages, esterase positive cells (*) and CD4+ T lymphocyte (flow cytometric analysis) in kidneys (n = 8 per group). *P < 0.05 compared to sham, **P < 0.05 compared to vehicle + I/R. B: F4/80, C: esterase staining, ×100.
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After CD45+ cells gating

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CD11c

sham  vehicle + I/R  FTY720 + I/R

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% CD11b+CD11c+ cells in CD11b+ cells

sham  vehicle+I/R  FTY720+I/R

**

B

CD80

sham  vehicle + I/R  FTY720 + I/R

% CD11c+CD80+ cells in spleen

sham  vehicle+I/R  FTY720+I/R

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Real time RT–PCR

For detection of FoxP3 mRNA expression, total RNA was purified by TRizol extraction (Invitrogen, Life Technologies, Korea) as described in the manufacturer’s protocol, and cDNA was synthesized using standard procedures. Real time RT–PCR was performed using an iCycler IQ Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA), the SYBR Supermix kit (Bio-Rad Life Science, Korea) and the RT2 PCR Primer Set for FoxP3 (Invitrogen, Life Technologies). The reference gene (RT2 PCR Primer Set, Applied Biosystems) used was 18s.

Statistical analysis

All data are presented as means ± SE and were analysed by Kruskal–Wallis tests. A P-value <0.05 was considered statistically significant.
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*After CD45+ cells gating*

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**Figure Description**

- **A** shows a flow cytometry scatter plot comparing Sham, Vehicle and FTY720 treatments. The x-axis represents the FL1-H (CD4 FITC) and the y-axis represents the FL4-H (FoxP3 APC). The histograms indicate the percentage of FoxP3+ cells in CD4+ cells for each group. Asterisk (*) indicates statistical significance.

- **B** displays the percentage of CD4+ cells that are FoxP3+ after CD45+ cells gating. The data is presented as a bar graph with error bars for each group, with the y-axis representing the percentage of CD4+ FoxP3+ cells and the x-axis representing Sham, Vehicle+I/R, and FTY720+I/R. Significant differences are indicated by asterisks.*
Results

FTY720 pretreatment attenuated I/R injury and was associated with peripheral lymphopaenia

As expected, pretreatment with FTY720 induced a marked peripheral lymphopaenia and significantly reduced both functional and histological evidence of kidney injury. Tubular cell apoptosis was also significantly reduced by FTY720 pretreatment (Figure 1). Pro-inflammatory cytokines (TNF-α, IL-6 and IFN-γ) and chemokines (MCP-1) were all significantly increased following I/R (data not shown), and pretreatment with FTY720 decreased the pro-inflammatory cytokine, IL-6, tissue levels. However, tissue levels of IL-10, an anti-inflammatory cytokine, were significantly increased in kidneys from FTY720-pretreated mice (Figure 2A). Tissue inflammation, assessed by immunohistochemical staining of monocyte/macrophages (F4/80+ cells) or neutrophils (esterase) and flow cytometric detection of CD4+ T cells, was significantly decreased in kidneys from FTY720-pretreated mice, confirming an anti-inflammatory effect of FTY720 (Figure 2B–D).

Fig. 4. Effect of FTY720 on Tregs. (A) The relative percentage of spleen CD4+FoxP3+ Tregs significantly increased in FTY720-treated mice. (B) The percentage and number of CD4+FoxP3+ Tregs in kidney were significantly increased in FTY720-treated mice. (C) Kidney FoxP3 mRNA expression was also markedly increased from FTY720-treated mice (n = 8 per group). *P < 0.05 compared to vehicle + I/R.

Fig. 5. Effect of FTY720 on conversion from non-regulatory T cells to Tregs. Splenocytes were incubated for 3 days with or without FTY720. (A) At 72 h, the percentage of CD4+FoxP3+ Tregs as well as FoxP3 mRNA expression was all increased in FTY720-treated cells (vehicle + IL-2 vs FTY720 + IL-2). (B) Brdu uptake was not different between the two groups. *P < 0.05 compared to vehicle + IL-2.
**FTY720 induced tolerogenic CD11b<sup>+</sup> CD11c<sup>+</sup> DCs**

In order to examine the effect of FTY720 pretreatment on DCs, the percentage of CD11b<sup>+</sup> CD11c<sup>+</sup> DC as well as their maturation status was analysed by flow cytometry. The percentage of CD11b<sup>+</sup> CD11c<sup>+</sup> DCs was increased in peripheral blood (Figure 3A), and a higher percentage of DCs showed mature phenotype (CD80<sup>+</sup> CD11c<sup>+</sup> cells) in FTY720-treated mice (Figure 3B), suggesting that FTY720 treatment may induce alterations in DC phenotypes (Figure 3B). To further address modulatory effects of FTY720 on DC immune function, we performed one-
way MLR. CD11c+ splenic DCs from FTY720-pretreated mice showed significantly impaired allostimulatory capacity compared to those from vehicle-treated mice, suggesting that FTY720 treatment may induce tolerogenic DCs (Figure 3C). However, while analysing kidney DC phenotypes, we found no difference in kidney CD11c+ DC phenotype (CD11b, CD80, MHC II) between the vehicle-treated and FTY720-treated mice despite significant increases in kidney CD11c+CD11b+ cell infiltration at 24 h following I/R (Figure 3D).

**FTY720 increased frequency of Tregs and FoxP3 mRNA expression and converted non-Tregs to Tregs**

To examine additional immune modulatory effects of FTY720, we first examined the frequency of Tregs in spleen and kidney from vehicle- or FTY720-treated ischaemic animals (vehicle + I/R vs FTY720 + I/R). The percentage of CD4+ FoxP3+ Tregs in spleen and kidney was significantly increased in FTY720-pretreated mice (Figure 4A, B). FoxP3 mRNA expression in kidney was markedly increased by FTY720 pretreatment (Figure 4C). Because FTY720 may induce a lesser retention of Tregs in lymph nodes than in conventional T cells, resulting in relative expansion in spleen and blood, we performed an additional study to further address whether FTY720 can induce conversion of Tregs from non-regulatory CD4+ cells in vitro. Splenocytes were incubated with FTY720-P/IL-2 or vehicle/IL-2. At 96 h, the percentage of CD4+ FoxP3+ Tregs as well as FoxP3 mRNA expression all increased in FTY720-treated splenocytes, suggesting that FTY720 directly induces proliferation of pre-existing Tregs or induces the conversion of naive T cells into Tregs (Figure 5A). However, the lack of BrdU incorporation into FTY720-treated splenocytes indicated that a conversion from non-regulatory cells to FoxP3 expressing Tregs, rather than proliferation of pre-existing Tregs, was responsible for the increase in CD4+ FoxP3+ cells in FTY720-treated splenocytes (Figure 5B).

**Depletion of Tregs reduced whereas adoptive transfer restored the beneficial effects of FTY720 in I/R-induced AKI**

Before the depletion study, we tested the efficacy of anti-CD25 mAb (PC61) treatment by measuring spleen Tregs. The percentage of CD4+ FoxP3+ Tregs decreased significantly (data not shown). Administration of PC61 mAb before FTY720 pretreatment also resulted in a partial depletion of Tregs in spleen and kidney, and this depletion was attenuated in FTY720-pretreated mice (Figure 6A). Systemic and kidney depletion of Tregs was associated with partial loss of the beneficial effects of FTY720 on kidney function (Figure 6B). To gain a better insight into a direct causal relationship, we performed an adoptive transfer experiment. Adoptive transfer of CD4+ CD25+ Tregs following Treg depletion partially restored the protective effect of FTY720, indicating that renoprotective effects of FTY720 are partially mediated by Tregs (Figure 7).

**Discussion**

In this study, we clearly demonstrated that the protective effect of FTY720 in I/R kidney injury was partially mediated by significant increases in the functional activity of CD4+ CD25+ FoxP3+ Tregs and possibly by DC modulation.

AKI is widely recognized as a disease having a high morbidity and mortality. Despite progress in understanding the pathophysiology of AKI, mortality still remains high [12,13]. Inflammation is known to play an important role in I/R injury, and accumulating evidence suggests the involvement of innate and adaptive immunity [14–20]. Although several new compounds having anti-inflammatory effects appear to be effective in reducing I/R injury, there is little evidence demonstrating a benefit of treatment in hu-
man AKI, partly due to the greater complexity of human AKI. For this reason, compounds that affect multiple pathways or a combination of drugs having different effects are expected to be more effective. FTY720, an analogue of S1P, has been shown to exert potent immunosuppressive activity and is effective in various autoimmune diseases and in organ transplantation [21–25]. Although FTY720 is thought to cause immuno-suppression mostly by sequestration of lymphocytes in secondary lymphoid organs, thereby inducing peripheral lymphopaenia with subsequent decreased tissue inflammation, several recent studies demonstrated that the drug may exert additional immune modulatory effects, such as DC modulation or induction of Treg activity [4–7]. CD4+ CD25+ FoxP3+ Tregs are important in the suppression of immune responses and in the maintenance of immune tolerance. They prevent the normal proliferation of CD4+ or CD8+ T cells via an IL-10/TGF-β-dependent fashion or via a direct cell contact-dependent mechanism, to thereby exert their immune suppressive function. In a Th1-mediated model of colitis, FTY720 significantly reduced histological evidence of colonic mucosal injury, and this effect was associated with marked upregulation of FoxP3 expression in colon tissue as well as in lamina propria [8]. Moreover, direct evidence that FTY720 can function to cause the conversion of conventional T cells to FoxP3+ Tregs was found in a model of virus-induced inflammatory disease caused by herpes simplex virus (HSV) infection [9]. In accordance with these findings, our study demonstrated that FTY720 pretreatment increased the relative percentage of Tregs and FoxP3 expression in spleen and kidney with a simultaneous attenuation of injury and inflammation during AKI. These data suggest that the reno-protective effects of FTY720 may be partly mediated through increased Tregs. The role that Tregs play in kidney I/R injury has recently been well demonstrated. For example, a lack of Tregs potentiated kidney injury and caused greater innate immune responses whereas Treg depletion partially attenuated the injury, confirming an important contribution of Tregs in reducing I/R injury [10]. In the same study, FoxP3+ Treg-deficient mice accumulated a greater number of inflammatory leukocytes [10]. In our study, we observed that increased mRNA transcription of Tregs and FoxP3 in FTY720-pretreated I/R kidneys was associated with a significantly decreased infiltration of neutrophils and macrophages, reduced pro-inflammatory cytokine and chemokine concentrations and increased anti-inflammatory cytokine IL-10. In addition, our finding of a many-fold increase in FoxP3 mRNA expression that was disproportionately high in FTY720-treated kidneys (more than 100-fold compared to vehicle), despite a very small but statistically significant increase in the percentage of Tregs, may suggest that FTY720 not only increased the number of Tregs but also potentiated FoxP3 activity in individual Treg cells.

To gain a better insight into the direct causal relationships between FTY720-induced Treg activity and its kidney protective effects, we used a strategy that caused Tregs’ depletion. The use of PC61 resulted in a more than 50% depletion of Tregs in spleen and kidney and was associated with partial loss of the reno-protective effect of FTY720. We also observed that adoptive transfer of Tregs following their depletion caused a restoration of their reno-protective effects. Altogether, these results suggest that a relative Treg expansion by FTY720 may directly contribute to the beneficial effects of FTY720. There are several possible mechanisms that contribute to the relative Treg expansion in FTY720-treated mice. First, the expansion may be related to the differential effect of FTY720 on the sequestration of Tregs compared to non-regulatory CD4 T cells. The density of S1P1 receptors in Tregs, which is known to be responsible for lymphocyte sequestration in secondary lymphoid organs, is known to be smaller than in conventional T cells. In addition, a previous study demonstrated that a reduced chemotactic response of Tregs to S1P, caused by FTY720, resulted in relative expansion of Tregs in the periphery [6]. A second mechanism of Treg expansion by FTY720 may be related to pre-existing Treg proliferation or conversion from naïve CD4+ T cells. Recently, several studies suggested that FTY720 can directly induce the conversion of T cells into FoxP3+ Tregs without involving cell trafficking [9,26]. We confirmed a possible conversion of non-Tregs to Tregs in our in vitro study. Comparable BrdU uptakes in FTY720- and vehicle-treated cells excluded the possibility that Tregs proliferated from pre-existing populations. In addition, we observed that TGF-β levels in cultured supernatants increased significantly in FTY720-treated wells (data not shown). Although it has been demonstrated that TGF-β plays an essential role in the expansion of FoxP3+ Treg cells [27,28], a blocking of TGF-β signalling in our study by anti-TGF-β monoclonal antibody did not diminish the percent of Tregs, suggesting that other mechanisms including downstream TGF-β signalling pathways may be responsible for the conversion of Tregs by FTY720. Indeed, others have shown that FTY720 shares a downstream signalling smad protein with TGF-β [29,30].

DCs are specialized antigen-presenting cells that link innate and adaptive immunity. Several reports recently demonstrated that DCs were able to stimulate immunologic tolerance by inducing tolerogenic DCs. S1P activation has also been demonstrated to modulate DC-mediated T cell responses to favour Th2 lymphocyte-dominant immunity. Muller et al. [5] showed that in vitro administration of FTY720 reduced the T cell stimulatory capacity of DCs and changed their cytokine production profile to promote Th2 differentiation. To elucidate a possible immune modulatory effect of FTY720 on DCs in ischaemic AKI, we first investigated the percent of myeloid DCs in peripheral blood and their phenotypes. FTY720 pretreatment resulted in significant increases in peripheral blood CD11b+ CD11c+ DC. It also increased the percentage of CD80+ mature DCs, suggesting the possibility that FTY720 can induce alterations in DC maturation status. However, DC function tested by one-way MLR assay showed that these cells were more likely tolerogenic than immunogenic because they showed a significantly impaired allostimulatory capacity. These observations contradict the classical theory that mature DCs generate immunity. However, several recent reports are in line with our observation that mature DCs can also induce immune tolerance. Constabel et al. appropriately demonstrated that CpG-induced DC maturation can reduce the uptake of exogenous Ags and/or lower the
processing and presentation of subsequently encountered Ags via major histocompatibility complex (MHC) class II molecules to CD4+ T cells [31]. DCs freshly isolated from the liver after I/R injury also exhibited a mature phenotype with an inhibitory profile that included increased IL-10 [32]. In addition, Kim et al. [33] recently found that a kidney DC subset showed a mature phenotype following I/R injury. The DCs were thought to show an anti-inflammatory phenotype, they secreted higher amount of IL-10, and contributed to the recovery process [33]. Therefore, it is currently believed that tolerogenic DCs can be defined only by their function rather than by specific lineage. Although we did not find a significant change in kidney DC phenotype in FTY720 pretreated mice, it is possible that expanded tolerogenic DCs may induce immune tolerance via secretion of anti-inflammatory cytokines, such as IL-10, or via indirect interactions with Tregs expansion.

**Conclusion**

Taken together, our study provides evidence that the protective effect of the SIP receptor analogue, FTY720, in I/R-induced AKI is partially mediated by increased Treg activity and possibly by DC modulation. Because the induction of tolerance is critical for the prevention of various inflammatory diseases including AKI, we expect that further studies identifying mechanisms of tolerance induction may be useful for developing various strategies for the prevention or treatment of AKI.

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**Conflict of interest statement.** None declared.

**References**


Dimethylarginine metabolism during acute and chronic rejection of rat renal allografts

Dariusz Zakrzewicz¹,* , Anna Zakrzewicz²,* , Sigrid Wilker² , Rolf-Hasso Boedeker³ , Winfried Padberg² , Oliver Eickelberg⁴ and Veronika Grau²

¹Department of Medicine II, University of Giessen Lung Center, Aulweg 123, D-35392 Giessen, Germany, ²Laboratory of Experimental Surgery, Department of General and Thoracic Surgery, Justus-Liebig-University Giessen, Rudolf-Buchheim-Str. 7, D-35385 Giessen, Germany, ³Institute for Medical Informatics, Section of Medical Statistics, Justus-Liebig-University Giessen, Heinrich-Buff Ring 44, D-35392 Giessen, Germany and ⁴Comprehensive Pneumology Center, Ludwig-Maximilians-University, Asklepios Hospital, and Helmholtz Zentrum München, Ingolstädter Landstr 1, D-85764 Neuherberg/München, Germany

Correspondence and offprint requests to: Veronika Grau; E-mail: veronika.grau@chiru.med.uni-giessen.de
*These authors contributed equally to this study.

Abstract

Background. Dimethylarginines are inhibitors of NO synthesis and are involved in the pathogenesis of vascular diseases. In this study, we ask the question if asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) levels change during fatal and reversible acute rejection, and contribute to the pathogenesis of chronic vasculopathy.

Methods. The Dark Agouti to Lewis rat strain combination was used to investigate fatal acute rejection. Fischer 344 kidneys were transplanted to Lewis rats to study reversible acute rejection episode and the process of chronic rejection. Isograft recipients and untreated Lewis rats were used as controls. L-arginine derivatives were determined by HPLC, and ADMA-metabolizing enzymes were studied by quantitative RT–PCR and western blotting.

Results. Renal transplantation transiently increased dimethylarginine levels independent of acute rejection. ADMA plasma levels did not importantly differ between recipients undergoing fatal or reversible acute rejection, whereas SDMA was even lower in recipients of Fisher 344 grafts. In comparison to isograft recipients, ADMA and SDMA levels were slightly elevated during reversible, but not during the process of chronic rejection. Increased dimethylarginine levels, however, did not block NO synthesis. Interestingly, protein methylation, but not ADMA degradation, was increased in allografts.

Conclusions. Our data do not support the concept that renal allografts are protected from fatal rejection by dimethylarginines. Dimethylarginines may play a role in triggering chronic rejection, but a contribution to vascular remodelling itself is improbable. In contrast, differential arginine methylation of yet unknown proteins by PRMT1 may be involved in the pathogenesis of acute and chronic rejection.

Keywords: ADMA; kidney transplantation; L-arginine; rat; SDMA

Introduction

Dimethylarginines have moved into the spotlight of scientific interest as endogenous inhibitors of nitric oxide synthesis. They are potential mediators of endothelial dysfunction, hypertension and vascular remodelling, and seem to be involved in chronic kidney diseases [1–7]. NO synthesis involves uptake of L-arginine (L-arg) by y⁺ transporters, which is inhibited by asymmetric dimethylarginine (ADMA) and by symmetric dimethylarginine (SDMA) [1,6]. These transporters are also needed for renal L-arg absorption and contribute to the maintenance of systemic L-arg levels. L-arg can be converted to NO and citrulline by NOS [2].

Dimethylarginine metabolism is also of outstanding interest in the context of transplantation, predominantly because the NOS isoforms, endothelial NOS (eNOS) and...