

Th17 Cells Are Not Directly Associated with Renal Ischemia–Reperfusion Injury

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Purpose: Interleukin-17-producing T cell (Th17 cell) is a newly discovered subtype of helper T cell. Its function and importance in the pathogenesis of a broad range of immune diseases are under active investigation. However, little is currently known about the role of Th17 cells in ischemia-reperfusion (IR) injury of the kidney, a common pathophysiologic occurrence in various renal disease processes.

Methods: We measured the number of infiltrated T lymphocytes and Th17 cells in C57Bl/6 mouse kidneys in sham-operated controls and following varying degrees of renal IR injury induced by renal pedicle clamping and reperfusion. The cell count results were compared to accompanying histologic damage and serum creatinine levels after 35 min and 45 min of ischemia, and following reperfusion of 48, 72, 96, and 168 hrs.

Results: The number of T lymphocytes increased as ischemia time increased. However, the number of Th17 cells was not significantly affected by prolonged ischemia and reperfusion. Furthermore, the degree of histologic damage and serum creatinine levels did not correlate with the T lymphocyte and Th17 cell count numbers.

Conclusion: We did not observe any evidence that Th17 cells are directly linked to renal tissue damage caused by IR injury. The role and importance of helper T cells in renal IR injury need to be evaluated further in the light of the interaction with Th1, Th2, and regulatory T cells rather than Th17 alone.

Key Words: Th17 cells, Acute kidney injury, Ischemia, Reperfusion injury

INTRODUCTION

CD4+ helper T cells are traditionally classified as Th1 and Th2 cells. Other subclasses of helper T cells were later discovered and named regulatory T cells (Tregs) and interleukin-17 (IL-17) producing T

cells (Th17 cells)¹. The pro-inflammatory characteristics of the IL-17 cytokine are well documented²; however, the role and importance of Th17 cells have only recently been discovered^{3, 4}.

The differentiation of Th17 cells from naïve T cell populations is initiated by the co-stimulation of IL-6 and transforming growth factor- β (TGF- β). Smad and STAT3 pathways, respectively, are used as signal transduction molecules and also eventually activate retinoic acid related orphan receptors γ t (ROR γ t). Th1 and Th2 cells inhibit this process through interferon- γ (IFN- γ) and IL-4⁵.

Diseases associated with T cell-mediated injury

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have previously been explained by the balance between Th1 and Th2 cells. However, after Th17 cell involvement was reported in experimental autoimmune encephalomyelitis⁶⁾, it was recommended that Tregs and Th17 cells should also be included in the explanation model of T cell-mediated injury.

Interleukin-17 produced by Th17 cells is a pro-inflammatory cytokine that induces neutrophil mobilization and differentiation⁷⁾ and T cell activation^{8, 9)}. However, functional studies on Th17 cells in specific organs and diseases have been inadequate and require further investigation. The relationship between Th17 cells and renal disease processes has mainly been concentrated on the study of kidney allograft rejection. It was suggested that IL-17 plays an important role in the early stages of allograft rejection by producing pro-inflammatory cytokines¹⁰⁾. In addition, Th17 cells also contribute to lymphoid neo-genesis in chronic rejection, thus deteriorating the clinical course¹¹⁾.

Ischemia-reperfusion (IR) injury of the kidney is a crucial pathophysiologic mechanism involved in acute renal failure and adverse drug reactions including cyclosporine toxicity as well as in allograft rejection. The infiltration of neutrophils and macrophages, activation of C5a and membrane attack complex, and activation of various cytokines have been thought to be involved in this type of injury. Recently, it was also revealed that T lymphocytes play a key role in IR injuries^{12, 13)}.

In this study, we used kidney ischemia-reperfusion injury in a mouse model to evaluate whether Th17 cells are responsible for the initiation and progression of this type of injury.

MATERIALS AND METHODS

1. Animals and experiments

All experiments were performed on 8-week-old male C57Bl/6 mice (Damul Science, Dajeon, Korea) weighing 25–26 g. They were provided standard

laboratory diet and water and cared under a protocol approved by the Institutional Animal Care and Use Committee of the Chungnam National University Medical School. We divided the mice into 3 groups: sham-operated mice (n=16, sacrificed 48hr, 72hr, 96hr, and 168hr post-operatively), 35 min ischemia reperfusion mice (n=14, sacrificed 48hr, 72hr, 96hr, and 168hr post-operatively), and 45 min ischemia reperfusion mice (n=14, sacrificed 48hr, 72hr, 96hr, and 168hr post-operatively). IR injury was induced by reperfusion after clamping (35 minutes and 45 minutes) both the renal artery and vein. Mice were anesthetized by IP injection (2.2 µl/g) with a mixture of ketamine (100 µg/g, Ketalar[®]; Bayer, Leverkusen, Germany) and xylazine (46 µg/g, Rompun[®]; Bayer).

2. Renal function

To evaluate renal function, blood was obtained via the inferior vena cava when the mice were sacrificed. Serum creatinine levels were evaluated using a Toshiba 200FR chemistry autoanalyzer (Toshiba Medical Systems Co., Tokyo, Japan).

3. Tissue preparation and light microscopy

Kidneys were excised immediately after sacrifice. One piece of the left kidney was fixed in 10% buffered formaldehyde at room temperature and then embedded in Paraplast (Sherwood Medical, St. Louis, MO, USA) for later histologic evaluation. Tissue sections (4 µm) were mounted on glass slides and stained with periodic acid-Schiff (PAS). Histologic grading of IR injury was performed as previously described¹⁴⁾ by one of the authors (Lim). Five serial fields along the outer medulla area were viewed under ×400 magnification. Necrosis, vacuolar change, and desquamation of tubular epithelial cells were regarded as histologic parameters of tubular damage, and the number of tubules showing damage in more than 50% of the epithelial cells was counted in each case.

4. Immunohistochemistry

Embedded tissues were cut serially (4 μm) and stained by an avidin–biotin complex method as previously described¹⁵. After deparaffinization and rehydration, antigen retrieval was performed using a microwave oven (2×5 min in citrate buffer, pH 6.0). A 0.3% H₂O₂ solution was used to block endogenous peroxidase activity. Primary antibodies, dilution, reaction time and temperature were as follows: CD3 (anti–mouse rabbit monoclonal, Lab Vision, Fremont Blvd., Fremont, CA, USA, 1:200, 2hr at room temperature) and IL–17 (anti–mouse rabbit polyclonal, Santa Cruz Biotechnology, CA, USA, 1:300, 2hr at room temperature). The number of cells exhibiting positive results with the above antibodies was counted in 5 serial high power fields (HPF) along the outer medulla area under ×400 magnification.

5. Statistical analysis

Data are presented as mean±standard deviation. Comparison between the groups was performed using the Kruskal–Wallis test or Mann–Whitney U test.

RESULTS

1. Histologic grading of IR injury and its relationship to serum creatinine levels

The sham–operated control group showed no evidence of renal damage. The 35 min IR group (n=14) showed tubular damage in 9.7±3.2% of the tubules compared to 13.8±4.2% in the 45 min IR group (n=14) and was statistically significant (p=0.003). The average serum creatinine level was also higher in the 45 min ischemia group (0.40±0.18 mg/dL in the 35 min ischemia group vs. 0.90±0.56 mg/dL in the 45 min ischemia group, and 0.20±0.03 mg/dL in the sham–operated group, p<0.01). The peak serum creatinine level was observed at 48hr reperfusion

time in both 35 min and 45 min ischemia groups (Fig. 1).

The extent of tubular damage was not proportional to reperfusion times in both the 35 min and 45 min ischemia groups. In the 35 min ischemia group, the percentage of tubular damage was not significantly affected by reperfusion times (11.6±4.4% at 48hr reperfusion time, 7.2±2.4% at 72hr, 8.2±1.3% at 96hr and 11.5±1.9% at 168 hr, p=0.113). In the 45 min ischemia group, tubular damage was observed in 13.6±3.4% of the tubules at 48hr reperfusion time, 15.3±3.1% at 72hr, 11.3±0.6% at 96hr and 15.7±8.1% at 168hr (p=0.349, Fig. 2).

2. Infiltration of T lymphocytes and Th17 cells

CD3–positive T lymphocytes were rarely observed (1.1±1.1/5HPF) and IL–17–positive Th17 cells were not observed in the kidneys of sham–operated mice. The number of T lymphocytes was not significantly different in the 35 min and 45 min ischemia groups (15.2±5.0/5HPF in 35 min vs. 15.4±2.9/5HPF in 45 min group, p=0.635). T lymphocyte infiltration was generally increased by the reperfusion time in the 35 min ischemia group (13.0±3.8/5HPF at 48hr, 10.7±2.3 at 72hr, 17.5±4.4 at 96hr, and 19.7±4.7 at 168hr,

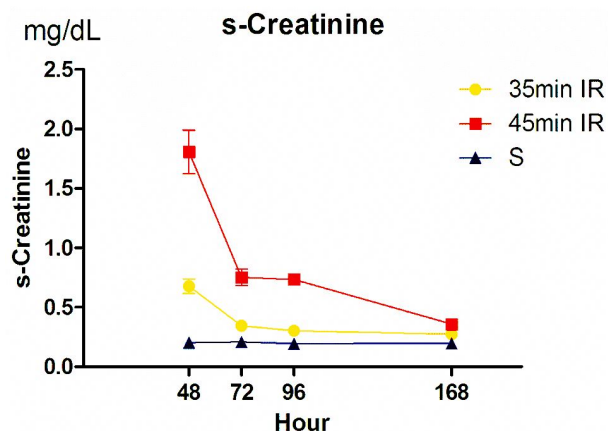


Fig. 1. Serum creatinine level. The average serum creatinine level was higher in the 45 min ischemia group (45 min IR) than in the 35 min ischemia group (35 min IR) or sham–operated mice (S).

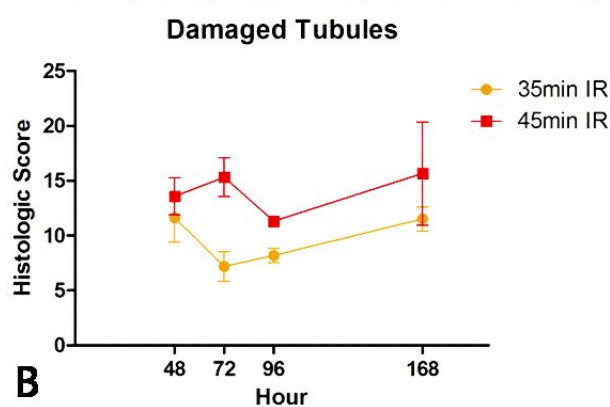
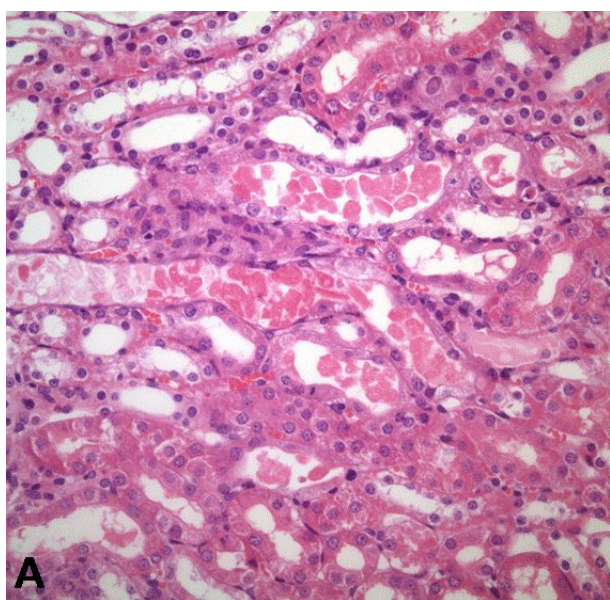


Fig. 2. Tubular damage after IR injury. Tubular epithelial cells showed necrosis, vacuolar change, and desquamation (A, PAS, original magnification $\times 200$). The 45 min ischemia group showed more severe damage than 35 min ischemia group. However, the extent of tubular damage was not proportional to reperfusion times in both the 35 min and 45 min ischemia groups (B).

$p=0.047$), however, there was no difference in the number of T lymphocytes in the 45 min ischemia group by the reperfusion times ($16.3 \pm 1.7/5\text{HPF}$ at 48hr, 16.3 ± 4.0 at 72hr, 16.3 ± 2.2 at 96hr, and 12.0 ± 2.0 at 168hr, $p=0.858$).

The number of Th17 cells was similar in the 35 min and 45 min ischemia groups ($2.8 \pm 1.3/5\text{HPF}$ in 35 min vs. $2.7 \pm 0.7/5\text{HPF}$ in 45 min group, $p=0.982$). The reperfusion times did not significantly affect the infiltration of Th17 cells in either group ($1.5 \pm 0.6/5$

HPF at 48hr, 3.0 ± 1.7 at 72hr, 3.3 ± 1.0 at 96hr, and 3.7 ± 0.6 at 168hr in the 35 min ischemia group, $p=0.079$ and $2.5 \pm 0.6/5\text{HPF}$ at 48hr, 2.3 ± 0.6 at 72hr, 3.0 ± 0.8 at 96hr, and 3.0 ± 1.0 at 168hr in the 45 min ischemia group, $p=0.561$, Fig. 3). The ratio of the number of Th17 cells to the number of CD3-positive T cells was similar in the 35 min and 45 min ischemia groups (0.19 ± 0.09 in 35 min vs. 0.18 ± 0.06 in 45 min, $p=0.839$). This ratio did not show significant change by the reperfusion times (0.13 ± 0.084 at 48hr, 0.28 ± 0.13 at 72hr, 0.20 ± 0.079 at 96hr, and 0.19 ± 0.050 at 168hr in the 35 min ischemia group, $p=0.296$ and 0.16 ± 0.048 at 48hr, 0.15 ± 0.042 at 72hr, 0.19 ± 0.057 at 96hr, and 0.25 ± 0.043 at 168hr in the 45 min ischemia group, $p=0.114$).

DISCUSSION

IR injury denotes the occurrence of tissue damage when blood supply returns to tissue after a period of ischemia. Although it is clear that the immune system is involved in IR injury of the kidney^{16, 17}), there is more to be investigated on the effector cells and the roles they play. The cellular components primarily associated with IR injury are those of the innate immune system such as neutrophils and macrophages. In this study, however, we evaluated the number of infiltrated T lymphocytes as there is growing evidence that adaptive immunity is also involved in IR injury¹⁸). As mentioned above, Th17 cell is a newly discovered subtype of T lymphocyte and our knowledge about its function and association with diseases is still expanding. Some investigators have presented evidence supporting Th17 cell involvement in renal IR injury. Tadagavadi et al. reported indirect evidence of this by showing that netrin-1, a neuronal guidance molecule, has a protective effect against renal IR injury through the inhibition of cytokines associated with the activation of Th1/Th2/Th17 cells¹⁹). Li et al. demonstrated that IL-17 plays an important role in the acute phase of renal IR injury as a component of innate immunity;

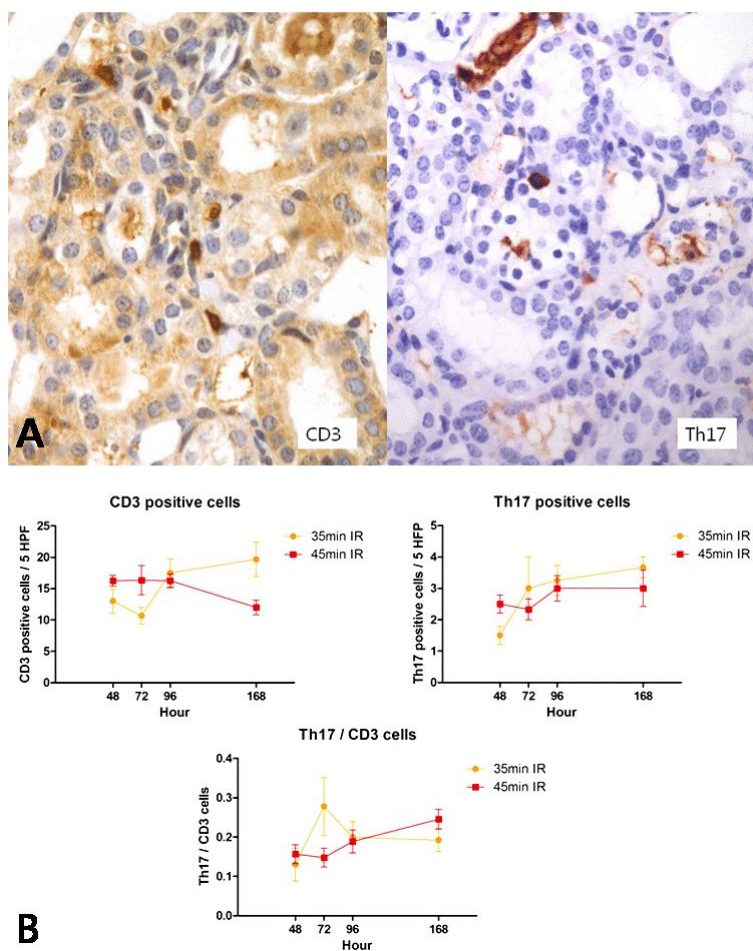


Fig. 3. CD3-positive T cell and Th17 cell infiltration after IR injury. Immunohistochemistry for CD3 and IL-17 revealed infiltration of T lymphocytes and Th17 cells (A, $\times 400$). The number of T lymphocytes was not significantly different in the 35 min and 45 min ischemia groups, and generally increased by the reperfusion time in the 35 min ischemia group. The number of Th17 cells was similar in the 35 min and 45 min ischemia groups. The reperfusion times did not significantly affect the infiltration of Th17 cells in either group. The ratio of Th17 cells and T lymphocytes showed similar pattern (B).

however, this interleukin was produced by infiltrating neutrophils, not by Th17 cells²⁰). Therefore, clear and direct evidence of Th17 cell involvement in renal IR injury has not yet been reported.

Based on our observations, the major determinant of the degree of tubular damage and serum creatinine levels was ischemia time rather than reperfusion time. The number of T lymphocytes and Th17 cells and the ratio were not affected by the length of ischemia time, and T lymphocyte number alone was increased by the prolongation of reperfusion in the 35 min ischemia

group. These results imply that the influence of T lymphocytes resulting in histologic damage during IR injury is not greater than those of other established contributing components such as neutrophils²¹) or the complement system²²). The role of lymphocytes in IR injury¹⁸) has recently been studied using CD4 and/or CD8 T cell-deficient mice. In these studies, histologic and serologic damage was ameliorated in CD4-deficient mice, while CD8-deficient mice showed similar damage as in the wild type mice. These results imply that helper T cells play a critical role in renal IR injury.

We also observed that while prolonged reperfusion times increased T cell infiltration, Th17 cells were not a major component of these infiltrates. Furthermore, histologic damage and serologic results were solely based on ischemia time, not T cell or Th17 cell infiltration.

In conclusion, we did not observe any evidence that Th17 cells are directly associated with renal tissue damage by IR injury. This study has some limitations, and the most important one is that quantitative and functional analysis of IL-17 was not performed. We only counted the number of infiltrating cells along the outer medulla and this method was used in our previous studies^{14, 23)}. However, we found limitations in employing this method as it neglected the cells in the inner medulla and cortex despite the small number of overall infiltrating cells. Although there are limitations as mentioned above in this study, it is reasonable to conclude that the role and importance of helper T cells in renal IR injury need to be further studied in the light of the interaction with Th1, Th2, and Tregs rather than Th17 alone.

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