The Korean Journal of Nephrology 2011; 30: 246~252

Original Article

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

Yoon Kyung Chang, M.D.[1], Dae Eun Choi, M.D.[2], Jin Young Jeong[2]
Hye Jin Choi[3] and Beom Jin Lim, M.D.[4]

Department of Internal Medicine[1], Daejeon St. Mary Hospital, Daejeon, Korea
Department of Internal Medicine[2] and Pathology[3],
Chungnam National University College of Medicine, Daejeon, Korea
Department of Pathology[4], Yonsei University College of Medicine, Seoul, Korea

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 documented[2]; 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 γt (RORγt). Th1 and Th2 cells inhibit this process through interferon-γ (IFN-γ) and IL-4[5].

Diseases associated with T cell-mediated injury

Submitted: 30 December 2010, Revised: 13 February 2011
Accepted: 16 March 2011
Correspondence: Beom Jin Lim, M.D.
Department of Pathology, Yonsei University College of Medicine, Gangnam Severance Hospital, 712, Eonjuro, Gangnamgu, Seoul, Korea, 135-720
Tel: 02)2019-3545, Fax: 02)3463-2103
E-mail: bjlim@yuhs.ac

have previously been explained by the balance between Th1 and Th2 cells. However, after Th17 cell involvement was reported in experimental autoimmune encephalomyelitis[6], 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 differentiation[7] 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 cytokines[10]. In addition, Th17 cells also contribute to lymphoid neo-genesis in chronic rejection, thus deteriorating the clinical course[11].

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 described[14] 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 described[15]. After deparaffinization and rehydration, antigen retrieval was performed using a microwave oven (2×5 min in citrate buffer, pH 6.0). A 0.3% H_2O_2 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,

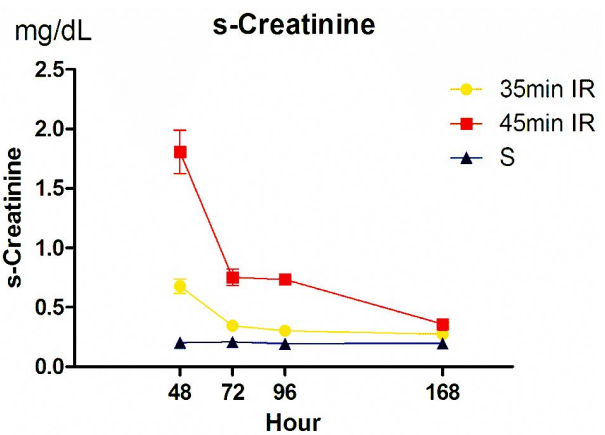

Fig. 1. Serum creatinine level. The average serum creatinine level was higher in the 45 min ischemia group (45 min IR) than in the 35 min ischemia group (35 min IR) or sham-operated mice (S).

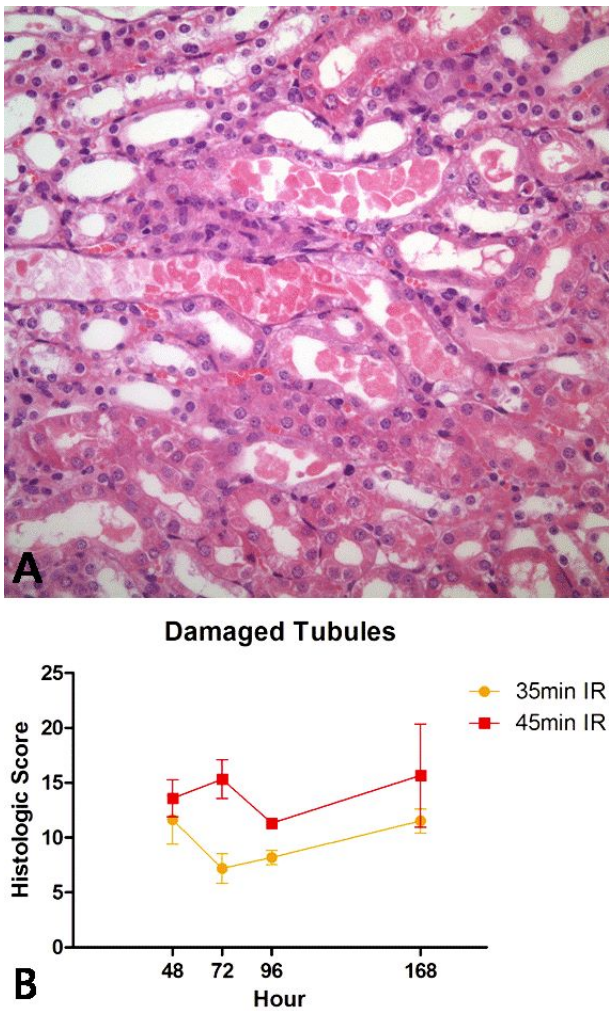

Fig. 2. Tubular damage after IR injury. Tubular epithelial cells showed necrosis, vacuolar change, and desquamation (**A**, PAS, original magnification ×200). The 45 min ischemia group showed more severe damage than 35 min ischemia group. However, the extent of tubular damage was not proportional to 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±1.7/5HPF 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±1.3/5HPF in 35 min vs. 2.7±0.7/5HPF in 45 min group, p=0.982). The reperfusion times did not significantly affect the infiltration of Th17 cells in either group (1.5±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±0.6/5HPF 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 injury[18]. 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 cells[19]. 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;

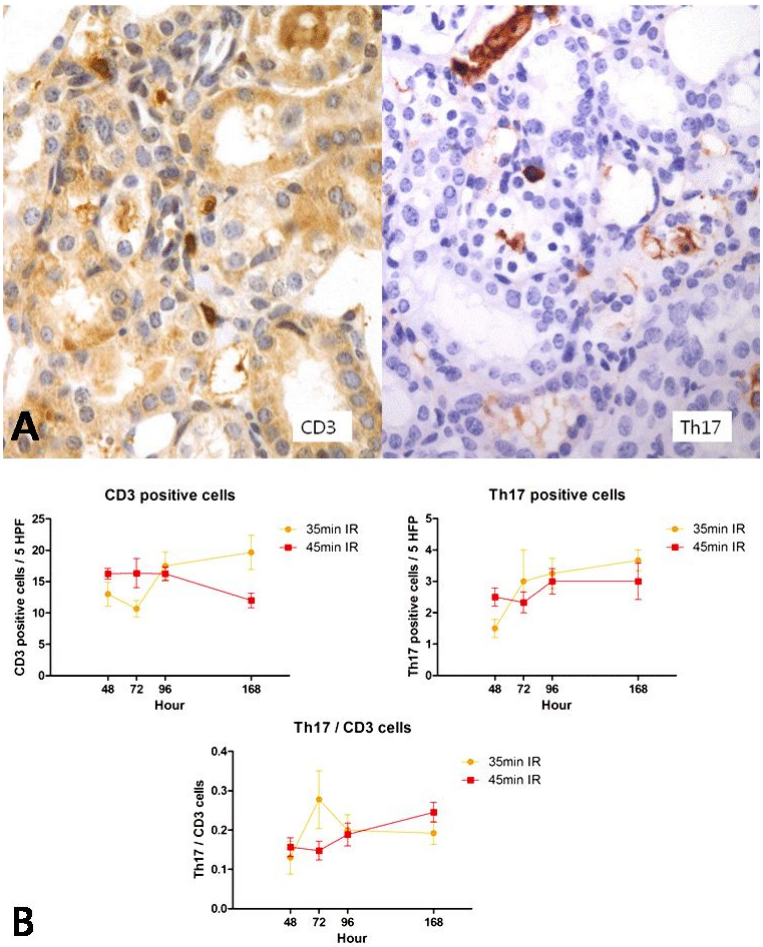

Fig. 3. CD3-positive T cell and Th17 cell infiltration after IR injury. Immunohistochemistry for CD3 and IL-17 revealed infiltration of T lymphocytes and Th17 cells (**A**, ×400). The number of T lymphocytes was not significantly different in the 35 min and 45 min ischemia groups, and generally increased 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 cells[20]. 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 neutrophils[21] or the complement system[22]. The role of lymphocytes in IR injury[18] 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.

ACKNOWLEDGEMENT

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (KRF-2008-331-E00044).

REFERENCES

1) Schmidt-Weber CB: Th17 and treg cells innovate the TH1/TH2 concept and allergy research. *Chem Immunol Allergy* 94:1-7, 2008
2) Kolls JK, Lindén A: Interleukin-17 family members and inflammation. *Immunity* 21:467-476, 2004
3) Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, Weaver CT: Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol* 6:1123-1132, 2005
4) Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, Wang Y, Hood L, Zhu Z, Tian Q, Dong C: A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol* 6: 1133-1141, 2005
5) Stockinger B, Veldhoen M: Differentiation and function of Th17 T cells. *Curr Opin Immunol* 19:281-286, 2007
6) Gocke AR, Cravens PD, Ben LH, Hussain RZ, Northrop SC, Racke MK, Lovett-Racke AE: T-bet regulates the fate of Th1 and Th17 lymphocytes in autoimmunity. *J Immunol* 178:1341-1348, 2007
7) Fossiez F, Banchereau J, Murray R, Van Kooten C, Garrone P, Lebecque S: Interleukin-17. *Int Rev Immunol* 16:541-551, 1998
8) Yu JJ, Gaffen SL: Interleukin-17: a novel inflammatory cytokine that bridges innate and adaptive immunity. *Front Biosci* 13:170-177, 2008
9) Afzali B, Lombardi G, Lechler RI, Lord GM: The role of T helper 17 (Th17) and regulatory T cells (Treg) in human organ transplantation and autoimmune disease. *Clin Exp Immunol* 148:32-46, 2007
10) Loong CC, Hsieh HG, Lui WY, Chen A, Lin CY: Evidence for the early involvement of interleukin 17 in human and experimental renal allograft rejection. *J Pathol* 197:322-332, 2002
11) Deteix C, Attuil-Audenis V, Duthey A, Patey N, McGregor B, Dubois V, Caligiuri G, Graff-Dubois S, Morelon E, Thaunat O: Intragraft Th17 infiltrate promotes lymphoid neogenesis and hastens clinical chronic rejection. *J Immunol* 184:5344-5351, 2010
12) Ascon DB, Lopez-Briones S, Liu M, Ascon M, Savransky V, Colvin RB, Soloski MJ, Rabb H: Phenotypic and functional characterization of kidney-infiltrating lymphocytes in renal ischemia reperfusion injury. *J Immunol* 177:3380-3387, 2006
13) Huang Y, Rabb H, Womer KL: Ischemia-reperfusion and immediate T cell responses. *Cell Immunol* 248:4-11, 2007
14) Choi DE, Jeong JY, Lim BJ, Na KR, Shin YT, Lee KW: Pretreatment with the tumor nerosis factor-alpha blocker etanercept attenuated ischemia-reperfusion renal injury. *Transplant Proc* 41:3590-3596, 2009
15) Lim BJ, Kim PK, Hong SW, Jeong HJ: Osteopontin expression and microvascular injury in cyclosporine nephrotoxicity. *Pediatr Nephrol* 19:288-294, 2004
16) Lim BJ, Hong SW, Jeong HJ: Renal tubular expression of Toll-like receptor 4 in cyclosporine nephrotoxicity. *APMIS* 117:583-591, 2009
17) Arslan F, Keogh B, McGuirk P, Parker AE: TLR2 and TLR4 in ischemia reperfusion injury. *Mediators Inflamm* 2010:704202, 2010
18) Linfert D, Chowdhry T, Rabb H: Lymphocytes and

ischemia-reperfusion injury. *Transplant Rev* (Orlando) 23:1-10, 2009
19) Tadagavadi RK, Wang W, Ramesh G: Netrin-1 regulates Th1/Th2/Th17 cytokine production and inflammation through UNC5B receptor and protects kidney against ischemia-reperfusion injury. *J Immunol* 185: 3750-3758, 2010
20) Li L, Huang L, Vergis AL, Ye H, Bajwa A, Narayan V, Strieter RM, Rosin DL, Okusa MD: IL-17 produced by neutrophils regulates IFN-gamma-mediated neutrophil migration in mouse kidney ischemia-reperfusion injury. *J Clin Invest* 120:331-342, 2010
21) Awad AS, Rouse M, Huang L, Vergis AL, Reutershan J, Cathro HP, Linden J, Okusa MD: Compartmentalization of neutrophils in the kidney and lung following acute ischemic kidney injury. *Kidney Int* 75:689-698, 2009
22) Diepenhorst GM, van Gulik TM, Hack CE: Complement-mediated ischemia-reperfusion injury: lessons learned from animal and clinical studies. *Ann Surg* 249: 889-899, 2009
23) Choi DE, Jeong JY, Lim BJ, Chung S, Chang YK, Lee SJ, Na KR, Kim SY, Shin YT, Lee KW: Pretreatment of sildenafil attenuates ischemia-reperfusion renal injury in rats. *Am J Physiol Renal Physiol* 297:F362-F370, 2009