original article

The role of Tregs and CD11c+ macrophages/dendritic cells in ischemic preconditioning of the kidney

Won Yong Cho[1], Hye Min Choi[1], So Young Lee[2], Myung Gyu Kim[1], Hyoung-Kyu Kim[1] and Sang-Kyung Jo[1]

[1]Division of Nephrology, Department of Internal Medicine, Korea University College of Medicine, Seoul, Korea and [2]Department of Internal Medicine, Eulji Medical School, Seoul, Korea

Dendritic cells have the potential to induce tolerance and here we attempted to identify their role in the tolerance seen in ischemic pre-conditioning. We induced bilateral renal ischemic preconditioning in mice and then challenged them with an ischemic insult 7 days later. Compared to sham-operated controls, preconditioned mice were found to have reduced injury with less inflammation, but had an increased number of regulatory T cells (Tregs) in their kidneys after the delayed insult. Splenocytes from these mice had more Tregs and mature CD11c+ cells, but reduced proliferative and cytokine-secretory responses, suggesting a state of immunosuppression compared to control mice. Anti-CD25 depletion followed by adoptive transfer of Tregs partially mitigated and then restored the protective effect of preconditioning. Depletion of CD11c+ cells with liposomes containing clodronate was associated with partial loss of preconditioning benefits. The increased numbers of Tregs or impaired immune response found in splenocytes from preconditioned mice were partially reversed in splenocytes from liposome clodronate–treated animals, suggesting that CD11c+ cells contribute to immune cell-mediated ischemic preconditioning. Hence, our results show that ischemic preconditioning of the kidney provides a negative signal to the peripheral immune system, partially mediating the tissue-protective and anti-inflammatory effects of this maneuver.

Correspondence: Sang-Kyung Jo, Division of Nephrology, Department of Internal Medicine, Korea University Anam Hospital, Korea University College of Medicine, 126-1, 5-Ka, Anam-Dong, Seongbuk-Ku, Seoul 136-705, Korea. E-mail: sang-kyung@korea.ac.kr

Ischemic preconditioning describes a phenomenon by virtue of which transient ischemia/reperfusion (I/R) injury to an organ confers a resistance to subsequent episodes of I/R injury and several mechanisms, such as induction of heme oxygenase-1, heat shock protein, or mitogen-activated protein kinase activation in ischemic organs, have been shown to be important.[1,2] However, several recent reports have demonstrated that the beneficial effect of ischemic preconditioning is limited not only to local tissue but also to remote organs, suggesting the presence of a systemic effect in ischemic preconditioning.[3,4] Since first reported in 1993 by Przyklenk et al.,[5] several recent randomized controlled trials have demonstrated the promising results of remote ischemic preconditioning (RIP) by utilizing limb ischemia to protect the myocardium or contralateral limb from subsequent I/R injury.[6–8] There are several proposed mechanisms of RIP, including neural and hormonal pathways, or the systemic immune response pathway with anti-inflammatory effect.[9] A recent report by Burne-Taney et al.[10] suggested the possible involvement of immune cells in mediating kidney ischemic preconditioning by showing the protective effect of adoptively transferred splenocytes of mice on I/R day 5 into nu/nu mice from subsequent I/R injury, and this immune cell participation may be one other possible mechanism leading to RIP. However, the precise phenotypic characteristics of peripheral immune system in tolerogenic status that might influence subsequent protection are not clear. Dendritic cells (DCs) that belong to monocyte lineage are specialized antigen-presenting cells with heterogenous phenotypes linking innate and adaptive immunity and are known to be important in induction of both immunity and tolerance. The DC lineage, maturational status, or cytokine milieu at sites of inflammation have all been implicated to be critical in determining whether an immunogenic or tolerogenic T-cell response will develop.[11,12] Therefore, given the striking functional plasticity of these cells, it is possible that DCs might play an important role in mediating tolerance induction in ischemic preconditioning. In the present study, under the hypothesis that immune cell alteration or immune suppressive signal following I/R might be important in

mediating kidney ischemic preconditioning effect, we examined the splenocytes' immunophenotypes, including the percentage of regulatory T cells (Tregs), phenotype of CD11c^{+} cells, and the proliferative and cytokine-secretory properties upon various stimuli. We also examined the role of Tregs in ischemic preconditioning by using anti-CD25 depletion and adoptive transfer strategies. In addition, we examined the role of CD11c^{+} cells by using liposome clodronate in an immune cell-mediated tolerance induction mechanism in kidney ischemic preconditioning. We observed that tolerogenic I/R day 7 splenocytes were characterized by an increased percentage of Tregs and more mature CD11c^{+} cells with tolerogenic phenotype. Kidney Tregs also increased in preconditioned ischemic kidneys, and depletion and adoptive transfer of Tregs was associated with partial loss and recovery of beneficial effect of ischemic preconditioning. Splenocytes from tolerogenic animals also showed a significantly decreased proliferative response upon T-cell receptor (TCR) stimulation and an impaired cytokine-secretory property upon lipopolysaccharide (LPS) stimulation. However, the phenotypic characteristics of these splenocytes were partially reversed by systemic CD11c^{+} cell depletion, and these changes were also associated with partial loss of beneficial effect of kidney ischemic preconditioning. Our results suggest that Tregs and/or CD11c^{+} macrophages/DCs might partially mediate immune cell-mediated tolerance induction in ischemic preconditioning. Further studies identifying the precise mechanisms of tolerance induction may hasten the development of various strategies in the prevention or treatment of acute kidney injury (AKI).

RESULTS

Delayed ischemic preconditioning attenuated the functional, histological kidney injury

As expected, delayed kidney ischemic preconditioning significantly attenuated the functional, histological kidney injury (Figures 1a and b). Compared with previous sham-operated ischemic kidneys with extensive tubular injury, characterized by tubular cell necrosis, dilation of tubules, and cast formation in the outer medulla, kidneys from preconditioned ischemic animals showed less tubular injury. The number of TdT-mediated dNTP nick end labeling-positive tubular cells was also markedly decreased by ischemic preconditioning (Figure 1c). Tissue inflammation, assessed by Ly6G immunohistochemical staining, also showed the anti-inflammatory effect of ischemic preconditioning. Ly6G-positive neutrophil infiltration observed along the course of outer stripes of the outer medulla in previous sham-operated ischemic kidneys markedly decreased in preconditioned ischemic kidneys (Figure 2a). Among several cytokines and chemokines, only interleukin (IL)-6 level showed a correlation with tissue inflammation, with significantly decreased tissue IL-6 levels in preconditioned ischemic kidneys (Figure 2b).

Tolerogenic status is associated with an increased percentage of spleen CD4^{+} Foxp3^{+} Tregs and percentage of kidney Tregs increased in preconditioned ischemic kidneys

In order to examine the mechanisms of immune cell-mediated tolerance mechanisms, we obtained splenocytes
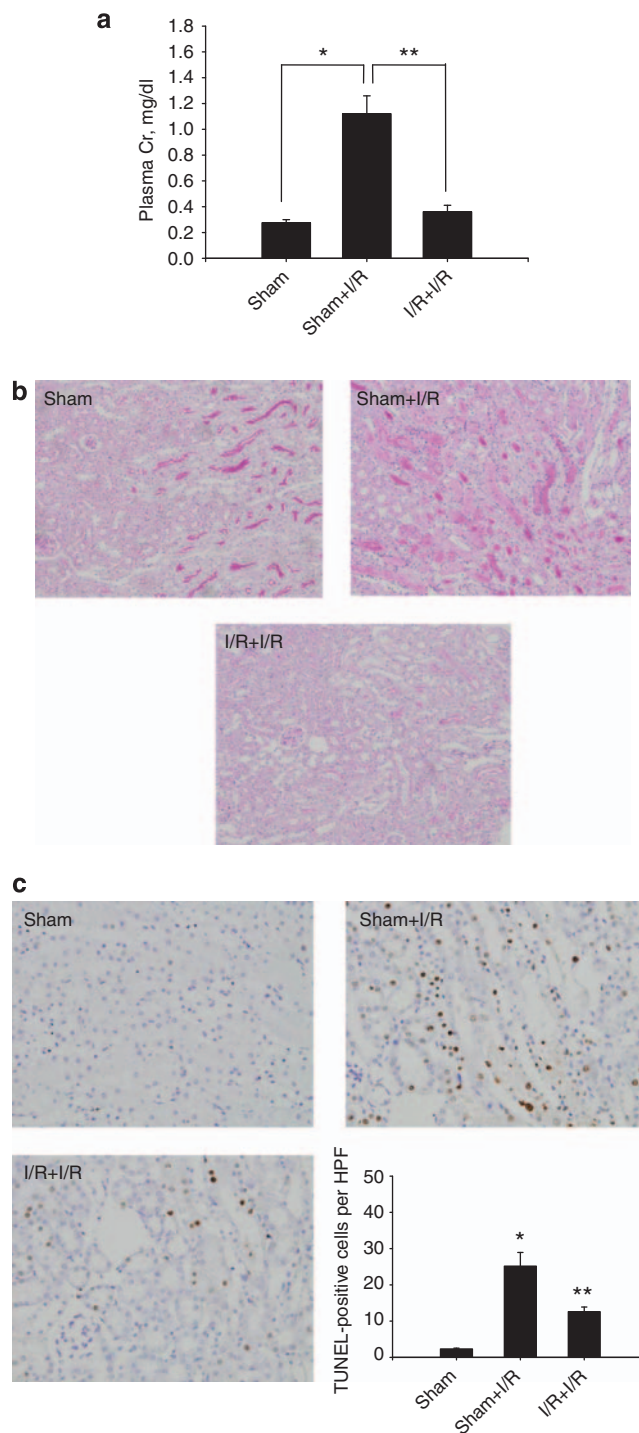
Figure 1 | Effect of delayed ischemic preconditioning on kidney function and histology. Following sham or ischemia/reperfusion (I/R) injury on day 0, a second I/R injury was done on day 7 and animals were killed for various analyses on day 8. (**a**) Serum creatinine (Cr) after second I/R. Serum Cr significantly decreased in preconditioned I/R + I/R mice. (**b**) Kidney histology, periodic acid-Schiff, × 40. (**c**) TdT-mediated dNTP nick end labeling (TUNEL)-positive cells, TUNEL staining, × 100. Tubular cell injury and apoptosis significantly decreased in I/R + I/R mice. *$P < 0.05$ compared with sham, **$P < 0.05$ compared with sham + I/R ($n = 5$–7 per group).
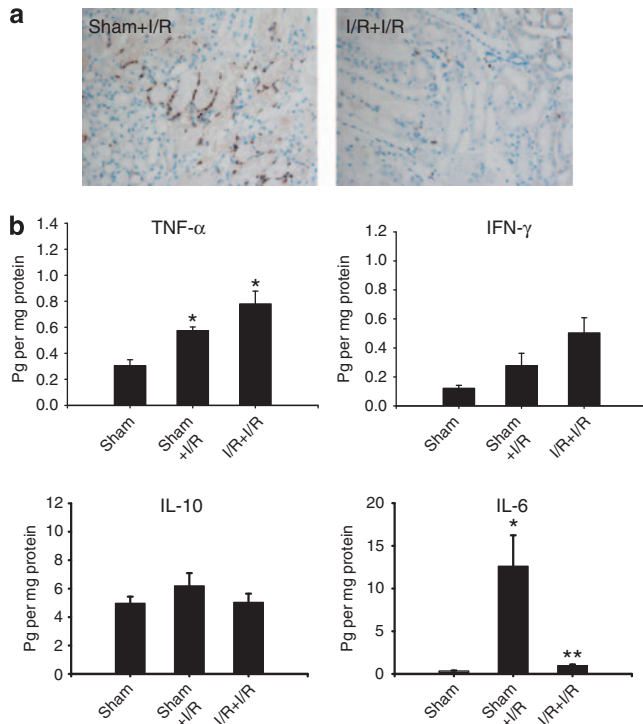
Figure 2 | **Effect of delayed ischemic preconditioning on kidney inflammation.** Following sham or ischemia/reperfusion (I/R) injury on day 0, a second I/R injury was done on day 7 and animals were killed for various analyses on day 8. (a) Immunohistochemistry for Ly6G, × 100. (b) Tissue cytokines by cytometric bead array. Ly6G$^+$ neutrophil infiltration into kidney tissue and kidney interleukin (IL)-6 level markedly increased in sham + I/R mice and significantly decreased in I/R + I/R. *$P < 0.05$ compared with sham, **$P < 0.05$ compared with sham + I/R ($n = 5$–7 per group). IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α.

from sham day 7 or tolerogenic I/R day 7 and examined the splenocyte immunophenotypes. We first determined the percentage of CD4$^+$ Foxp3$^+$ Tregs in the spleen that had been known to be important in tolerance induction in various autoimmune and transplantation animal models. The percentage of splenocyte CD4$^+$ Foxp3$^+$ Tregs was slightly but significantly increased in tolerogenic I/R day 7 mice compared with sham-operated animals ($7.36 \pm 0.52\%$ vs $5.29 \pm 0.12\%$ from CD4$^+$ cells, $P < 0.05$; Figure 3a). In addition, we checked the percentage of kidney Tregs 24 h after second I/R. Compared with sham + I/R, the percentage of CD4$^+$ Foxp3$^+$ Tregs increased significantly in kidneys from I/R + I/R (Figure 3b).

Tregs partially contribute to the protective effect of ischemic preconditioning

To determine the functional significance of increased Treg population in ischemic preconditioning, we used depletion and adoptive transfer strategy. Administration of anti-CD25mAb significantly decreased the number of splenic Tregs and the Treg depletion was accompanied by partial loss of preconditioning effect. In addition, we observed that the

adoptive transfer of these cells back into Treg-depleted mice partially restored the protective effect of ischemic preconditioning (Figures 3c and d), suggesting the important contribution of Tregs in ischemic preconditioning. Transferred Tregs were identified in the spleen, lymph nodes, and also in kidneys (Figure 3e), but not in the liver and lungs (data not shown). The intensity of GFP-positive cells can be appreciated by 4′,6-diamidino-2-phenylindole counterstaining.

Decreased proliferation and impaired cytokine secretion of splenocytes in tolerogenic I/R day 7 animals

For further analyses of splenocyte function, we measured the proliferative response of splenocytes upon TCR stimulation and also the cytokine-secretory properties upon LPS stimulation. Compared with splenocytes from sham-operated animals that showed proliferation upon combined anti-CD3 and anti-CD28 antibody (Ab) stimulation, splenocytes from I/R day 7 mice showed a significantly decreased proliferative response determined by bromodeoxyuridine (BrdU) incorporation (Figure 4a). In addition, the splenocyte cytokine-secretory properties upon LPS stimulation were also measured. LPS simulation markedly increased the production of tumor necrosis factor-α, interferon-γ, and monocyte chemotactic protein-1 in the splenocytes of sham-operated animals. However, splenocytes from I/R day 7 mice showed significant impairment in secretion of proinflammatory cytokines and chemokines upon LPS stimulation (Figure 4b).

CD11c$^+$ cells from I/R day 7 animals show different phenotype

A higher percentage of CD11c$^+$ cells in the I/R day 7 mice expressed co-stimulatory molecules compared with those from sham-operated animals (Figure 5a). Among cytokines, IL-10 and IL-6 levels were significantly higher in CD11c$^+$ cells from I/R day 7 mice compared with those from sham-operated mice (Figure 5b), suggesting that CD11C$^+$ cells in I/R day 7 mice were in a more mature stage compared with those in sham-operated mice. However, no detectable IL-12 p70 was found in DCs from tolerogenic animals, possibly suggesting that they reside in semi-mature DCs that are known to induce tolerance by promoting differentiation of Tregs from naïve CD4$^+$ T cells (Figure 5a).

Liposome clodronate injection resulted in systemic depletion of CD11c$^+$ CD11b$^+$ cells and was associated with partial loss of the beneficial effect of ischemic preconditioning

Liposome clodronate injection following initial I/R resulted in significant depletion of CD11b$^+$ CD11c$^+$ cells in peripheral blood and spleens. Depletion of CD8$^+$ CD11c$^+$ cells that are known to be lymphoid DCs was also observed (Figure 6).

Systemic depletion of CD11c$^+$ cells before second I/R was associated with partial loss of the beneficial effect of kidney ischemic preconditioning on kidney function and histology. The decreased serum creatinine level and tubular damage observed in preconditioned ischemic kidneys showed a
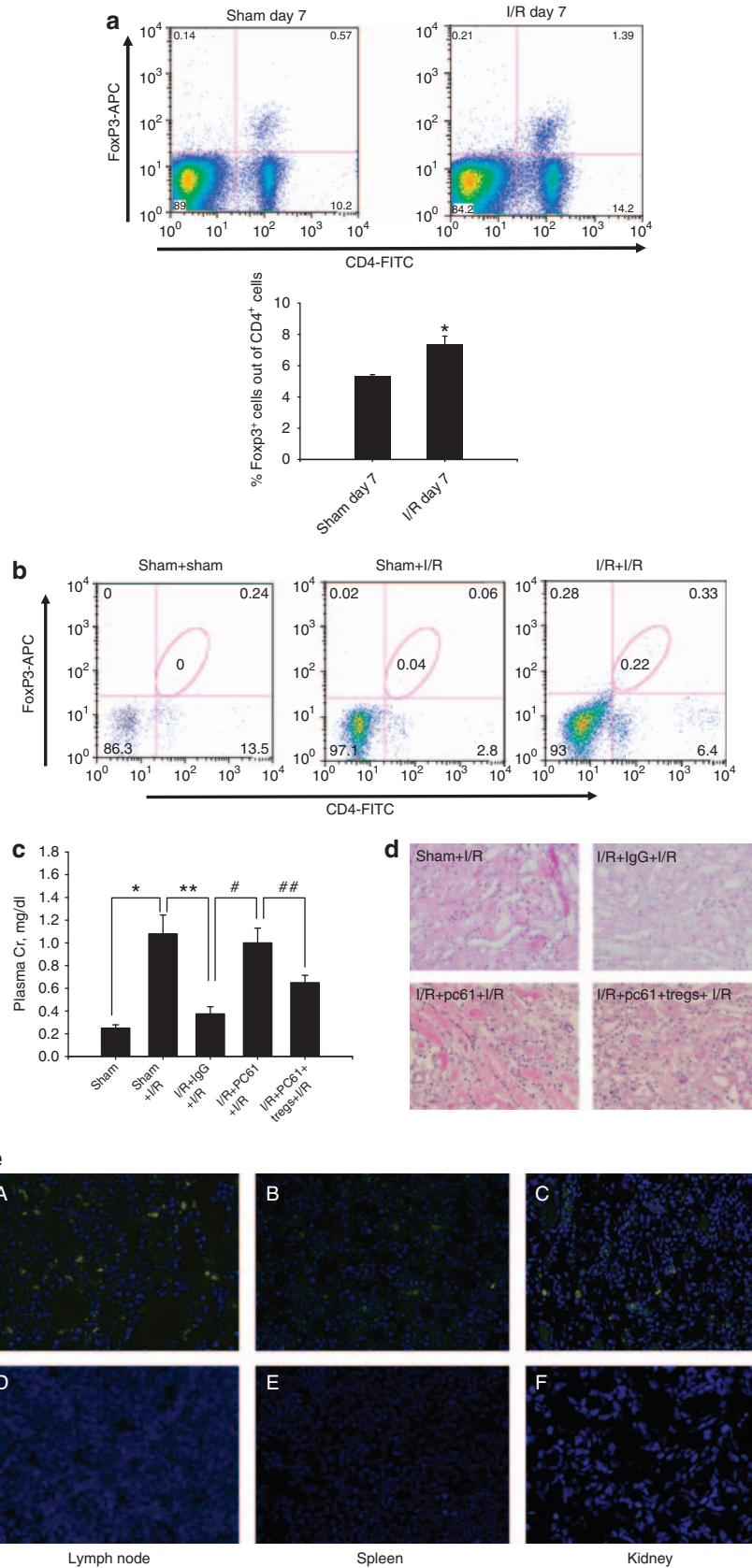
partial reversal by liposome clodronate injection following initial I/R (Figures 7a and b). The anti-inflammatory effect of ischemic preconditioning also partially disappeared by liposome clodronate injection (Figure 7c), and the markedly decreased tissue IL-6 level in preconditioned ischemic kidney also showed a partial reversal, suggesting that the beneficial effect of kidney ischemic preconditioning might be partially mediated by CD11c$^+$ macrophages/DCs. As IL-10 has been known to be associated with DC–Treg interaction, we measured the kidney IL-10 level and also performed immunohistochemical localization. IL-10-positive cells were mainly localized to the inner medulla and papilla. Compared with sham-operated kidneys, the number and staining intensity of IL-10-positive cells in papilla seem to increase

in I/R day 7 kidneys. However, the difference between I/R day 7 and I/R + liposome clodronate day 7 is not remarkable (Figure 7d). IL-10 protein level from total kidney extracts also showed no significant difference between the two groups (Figure 7e).

Splenocyte phenotypes in tolerogenic animals were partially reversed by liposome clodronate injection

In the analysis of splenocyte phenotypes, the increased percentage of CD4$^+$ Foxp3$^+$ Tregs, or decreased proliferative response upon TCR stimulation and impaired cytokine-secretory properties upon LPS stimulation, which were all observed in splenocytes from tolerogenic animals, were partially reversed in liposome clodronate-injected preconditioned animals, suggesting the important role of DC/macrophages in mediating peripheral immune suppressive signal following I/R (Figure 8).

DISCUSSION
In the present study, we demonstrated the following: (i) delayed kidney ischemic preconditioning provided marked functional and histological kidney protection with less tissue inflammation; (ii) splenocytes from the tolerogenic I/R day 7 state were characterized by an increased percentage of Tregs, significantly decreased proliferative response upon TCR stimulation, and also impaired cytokine-secretory property upon LPS stimulation; (iii) depletion and adoptive transfer of Tregs partially mitigated and restored the protective effect of ischemic preconditioning; (iv) CD11c$^+$ macrophages/DCs from the tolerogenic I/R day 7 state are thought to be in a more mature stage with high surface expression of co-stimulatory molecules, higher IL-6 and IL-10 secretion; (v) systemic DC/macrophage depletion by liposome clodronate injection following I/R was associated with partial loss of the beneficial effect of ischemic preconditioning; and (vi) increased Tregs or less proliferative and cytokine-secretory response, all observed in splenocytes from tolerogenic I/R day 7 state, showed a partial reversal by liposome clodronate injection. These results demonstrated that ischemic preconditioning induced peripheral immune tolerance status that is characterized by expansion of Tregs and more mature CD11c$^+$ cells and thus might suggest that Tregs or macrophage/
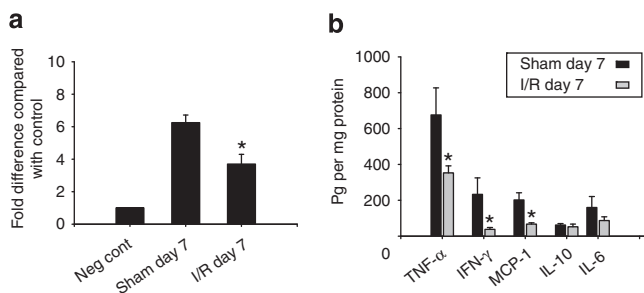
Figure 4 | Splenocytes of tolerogenic ischemia/reperfusion (I/R) day 7 animals showed decreased proliferative response upon mitogenic stimuli, and impaired secretion of cytokines and chemokine-secretory response upon lipopolysaccharide (LPS) stimulation. Following sham or I/R injury on day 0, animals were killed on day 7 and mononuclear cells from spleen were obtained for various analyses. (**a**) Spleen mononuclear cells from sham day 7 or I/R day 7 were cultured with or without anti-mouse CD3 antibody (Ab)/anti-mouse CD28 Ab for 72 h and, following incubation with bromodeoxyuridine (BrdU) for 18 h, the amount of BrdU incorporation was measured. Compared with sham-operated mice, spleen MNCs from I/R day 7 showed significantly decreased proliferative response. (**b**) Spleen MNCs from sham day 7 or I/R day 7 mice were cultured in the presence of LPS for 24 h and cytokine and chemokine production was measured in the supernatants. Compared with sham-operated mice, spleen MNCs from I/R day 7 mice showed significantly impaired cytokine secretion. *$P < 0.05$ compared with sham day 7 ($n = 5$–6 per group). IFN-γ, interferon-γ; IL, interleukin; MCP-1, monocyte chemotactic protein-1; TNF-α, tumor necrosis factor-α.

Figure 3 | CD4$^+$ Foxp3 regulatory T cells (Tregs) contribute to the beneficial effect of ischemic preconditioning. (a) Following sham or ischemia/reperfusion (I/R) injury on day 0, mice were killed on day 7 and mononuclear cells from spleen were obtained for various analyses. Flow-cytometric detection of CD4$^+$ Foxp3$^+$ Tregs and percentage of Tregs out of the total number of CD4$^+$ cells. The percentage of spleen Tregs increased in tolerogenic I/R day 7 mice. (**b**) Following sham or I/R injury on day 0, a second I/R injury was done on day 7 and animals were killed on day 8. Flow-cytometric detection of kidney CD4$^+$ Foxp3$^+$ Tregs in sham, sham + I/R, and I/R + I/R mice was done. The percentage of kidney Tregs increased in preconditioned ischemic kidneys (I/R + I/R) compared with sham + I/R. (**c**) Serum creatinine (Cr) after second I/R in Treg-depleted or adoptively transferred mice. Treg depletion by using CD25mAb (PC61) or adoptive transfer of CD4$^+$ Foxp3$^+$ GFP$^+$ cells partially mitigated or restored the protective effect of ischemic preconditioning. *$P < 0.05$ compared with sham day 7, **$P < 0.05$ compared with sham + I/R, #$P < 0.05$ compared with I/R + I/R, ##$P < 0.05$ compared with I/R + PC61 + I/R, $n = 4$–5 per group. (**d**) Kidney histology showed loss of protective effect of ischemic preconditioning in Treg-depleted mice and also partial reversal of injury in mice with adoptive transfer of Tregs, periodic acid-Schiff, × 100. (**e**) Detection of adoptively transferred Tregs in various tissues. Mice were treated with CD4$^+$ GFP$^+$ Tregs from Foxp3^{EGFP} mice (A–C) or phosphate-buffered saline (D–F) on day 2 following Treg depletion after initial I/R as described in Methods, and tissues were processed for immunofluorescence microscopy 1 day after the second I/R. All pictures were counterstained with 4′,6-diamidino-2-phenylindole. Original magnification, × 400. (A, D) Lymph node; (B, E) spleen; (C, F) kidney. IgG, immunoglobulin G.
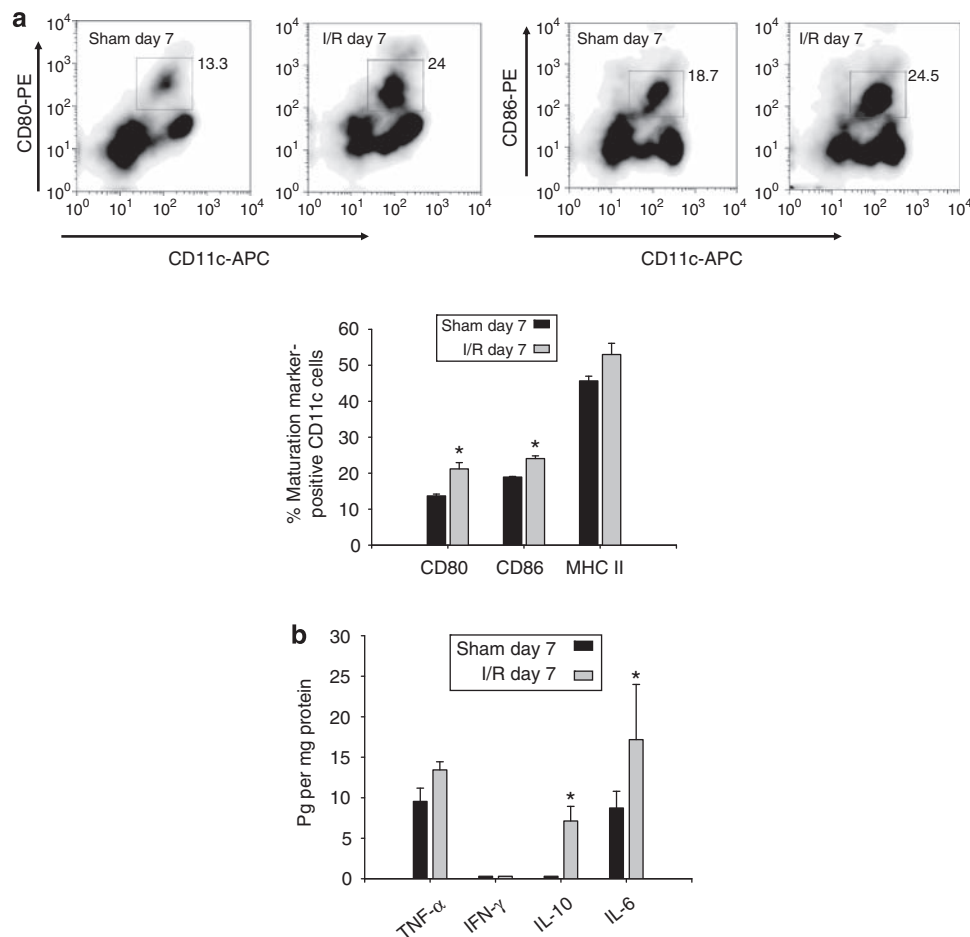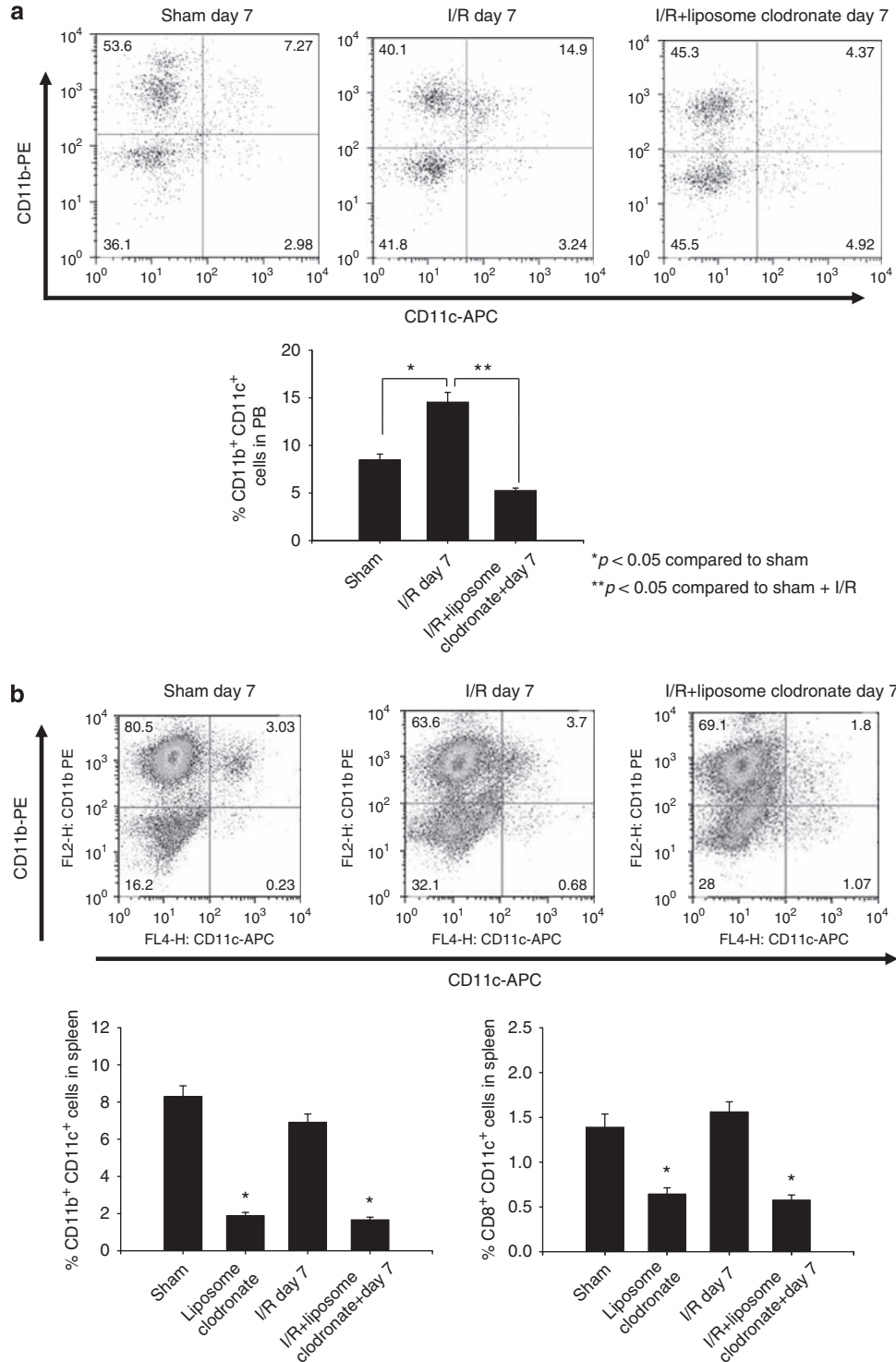
Figure 5 | CD11c$^+$ spleen macrophages/dendritic cells (DCs) in tolerogenic state are in their semi-mature stage. Spleen CD11C$^+$ DCs were separated by using magnetic-assisted cell sorting (MACS) and flow-cytometric detection of their maturation status and cytokine production was examined. (**a**) Percentages of CD80- or CD86-expressing CD11c$^+$ spleen DCs were measured. The percentage of CD11c$^+$ cells that express the activation marker was significantly increased in ischemia/reperfusion (I/R) day 7 mice. (**b**) Spleen CD11c$^+$ DCs were cultured overnight in 96-well plates and their cytokine production was measured. Secretion of interleukin (IL)-6 and IL-10 was significantly increased in CD11c$^+$ cells from I/R day 7 animals compared with those from sham day 7 animals. *$P < 0.05$ compared with sham day 7 ($n = 4$). IFN-γ, interferon-γ; MHC II, major histocompatibility complex II; TNF-α, tumor necrosis factor-α.

DC-mediated immune suppression is thought to contribute to tolerance induction in ischemic preconditioning.

Ischemic preconditioning represents an endogenous adaptive response that provides local protection against subsequent I/R injury and the beneficial effect of ischemic preconditioning is thought to result from local activation of mitogen-activated protein kinase, induction of heat shock protein, or inducible nitric oxide synthase.[1,2,13] However, recent observations that the beneficial effect of ischemic preconditioning is limited not only to local tissues but also to remote organs suggests that the preconditioning effect might also be systemically mediated.[3,4] Among the several proposed mechanisms of RIP, a systemic anti-inflammatory response following I/R that is associated with immune cell alteration might be an attractive, novel mechanism. Burne-Taney et al. previously reported the possible involvement of immune cells in kidney ischemic preconditioning by showing the protective effect of adoptively transferred splenocytes isolated

from I/R day 5 animals to nu/nu mice from subsequent I/R injury compared to those from sham day 5 animals.[10] In our study, we focused on the peripheral immune response in preconditioned mice to examine the possible contribution of systemic immune response in ischemic preconditioning. We observed that the tolerogenic I/R day 7 state was associated with peripheral immune-suppressive signals that were characterized by an increased percentage of Tregs and CD11c$^+$ macrophages/DCs with more mature phenotype. A depressed peripheral immune system following I/R injury to other organs has also been reported. Offner et al.[14] showed that early activation of systemic immunity was followed later by profound immunosuppression characterized by increased CD4$^+$ FoxP3$^+$ Tregs in an ischemic stroke mouse model. They claimed that this profound peripheral immunosuppression might result in increased infectious complications, which is a frequent medical complication diagnosed in stroke patients. In another study, Konstantinov et al.[15] also

Figure 6 | **Effect of liposome clodronate administration after initial ischemia/reperfusion (I/R) injury on blood and spleen CD11b$^+$ CD11c$^+$ macrophages/dendritic cells (DCs).** Liposome clodronate was injected on days 1 and 3 following initial I/R injury, and on day 7 mice were killed. Flow-cytometric detection of CD11b$^+$ CD11c$^+$ DCs or CD8$^+$ CD11c$^+$ DCs was performed. (**a**) After gating monocytes, flow-cytometric detection of CD11b$^+$ CD11c$^+$ DCs in blood was performed. Depletion of CD11b$^+$ CD11c$^+$ cells compared with CD11b$^+$ CD11c$^-$ cells was evident. (**b**) Flow-cytometric analyses of spleen CD11b$^+$ CD11c$^+$ and CD8$^+$ CD11c$^+$ cells. More preferential depletion of CD11b$^+$ CD11c$^+$ compared with CD8$^+$ CD11c$^+$ is evident. *$P < 0.05$ compared with sham day 7, **$P < 0.05$ compared with I/R day 7 ($n = 4$–5). APC, allophycocyanin; LC, liposome clodronate.
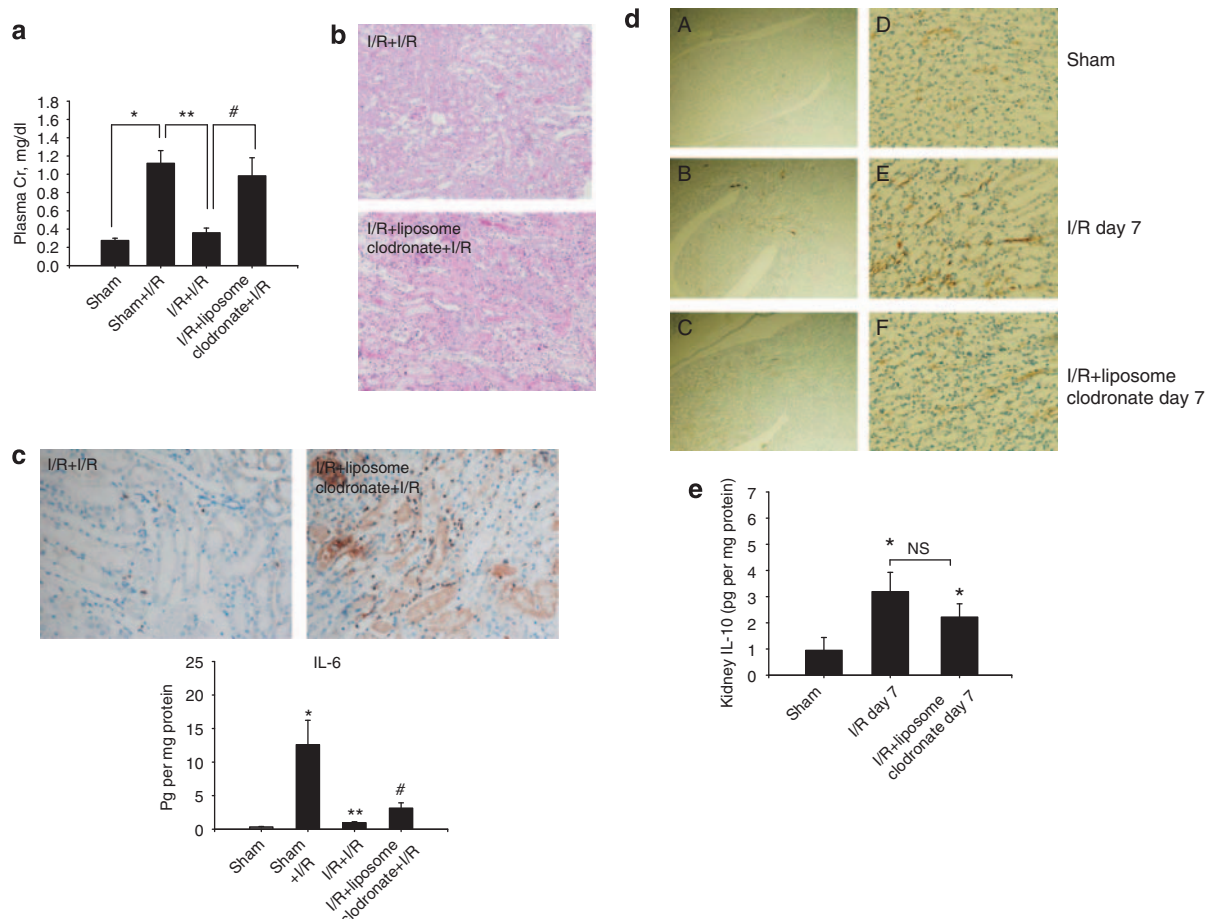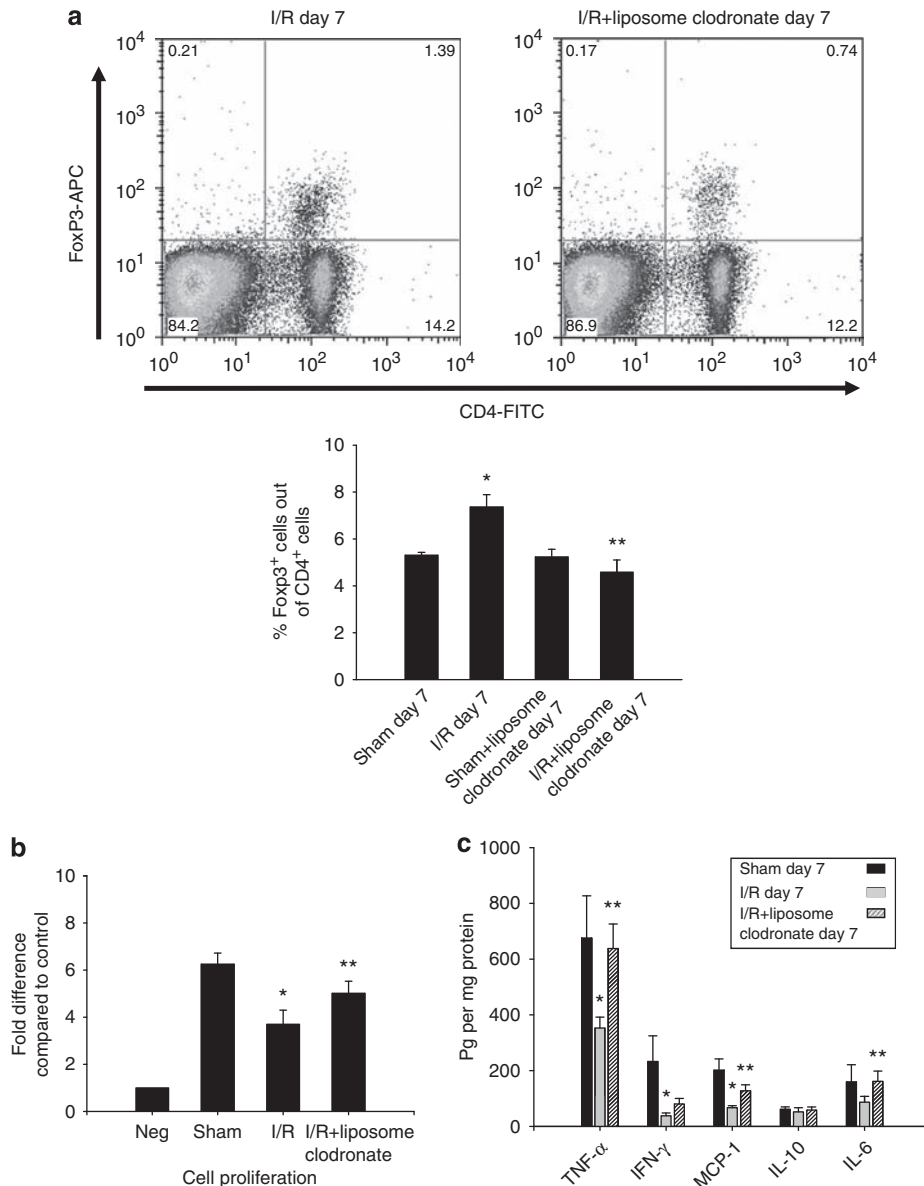
Figure 7 | Effect of liposome clodronate administration on delayed kidney ischemic preconditioning. Following sham or ischemia/reperfusion (I/R) injury on day 0, a second I/R injury was done on day 7 and animals were killed for various analyses on day 8. In the I/R + liposome clodronate + I/R group, liposome clodronate was injected intravenously on days 1 and 3 following initial I/R, and on day 7 a second I/R injury was done. (**a**) Serum creatinine (Cr) after second I/R. (**b**) Kidney histology, periodic acid-Schiff, × 100. (**c**) Ly6G immunohistochemistry, × 200. The beneficial effect of ischemic preconditioning on kidney function, histology, and inflammation was partially reversed in I/R + liposome clodronate + I/R mice. (**d**) Interleukin (IL)-10 immunohistochemistry. (A, D) Sham day 7, (B, E) I/R day 7, (C, F) I/R + LC day 7. (A–C) × 40, (D–F) × 200. (**e**) Kidney IL-10 level measured by cytometric bead array, *$P < 0.05$ compared with sham, **$P < 0.05$ compared with sham + I/R, #$P < 0.05$ compared with I/R + I/R ($n = 5$–6) per group. NS, non-significant.

demonstrated suppression of inflammatory gene transcription in circulating leukocytes following brief forearm ischemia using microarray methods. The suppressed genes in that study were those involved in cytokine synthesis, leukocyte chemotaxis, adhesion or migration, and innate immunity signaling pathways. Transcriptosome change was evident within 15 min (early-phase IPC) or 24 h (delayed-phase IPC) following ischemia.[15] This change in immune cells toward impaired activation is likely to contribute to less inflammation and organ injury upon subsequent I/R because tissue inflammation has been known to be very important in pathogenesis of I/R-induced AKI.

CD4+ CD25+ Tregs are important in suppression of immune responses and also in induction of tolerance in various injury models, and recently several reports have demonstrated a beneficial effect of CD4+ CD25+ cells in Th-1-mediated diseases by using Treg depletion strategies.[16–20] We observed that the relative percentage of Tregs in spleen increased in tolerogenic I/R day 7 mice. Although the magnitude of increase in splenic Tregs in I/R day 7 mice or increase in kidney Tregs in I/R + I/R mice was small, the role of Tregs in ischemic preconditioning was proved to be important because partial depletion and adoptive transfer of Tregs partially mitigated and restored the beneficial effect of ischemic preconditioning. Recently, the protective role of Tregs in kidney I/R injury has been reported. By using three different animal models to modulate Tregs, Kinsey et al.[21] demonstrated that Tregs have a protective role in I/R injury through IL-10-mediated suppression of innate immunity. Gandolfo et al.[22] also reported that Tregs have an important role in recovery phase following I/R. They also observed significantly increased trafficking of Tregs into the kidney at 3 and 10 days after I/R, respectively. Therefore, Tregs are likely to contribute not only to the repair process in I/R injury but also to the tolerance induction mechanism upon subsequent injury.

Figure 8 | Effect of liposome clodronate administration on splenocyte phenotypes. Following sham, or ischemia/reperfusion (I/R) injury on day 0, animals were killed on day 7 and mononuclear cells from spleen were obtained for various analyses. In the I/R + liposome clodronate group, liposome clodronate was injected intravenously on days 1 and 3 following initial I/R. (**a**) Percent CD4^{+} Foxp3^{+} regulatory T cells (Tregs) out of the total number of CD4^{+} cells. (**b**) Proliferative response of splenocytes determined by bromodeoxyuridine incorporation upon anti-mouse CD3 antibody (Ab) and anti-mouse CD28 Ab stimulation for 72 h. (**c**) Cytokine-secretory response of splenocytes upon lipopolysaccharide stimulation for 24 h. *$P < 0.05$ compared with sham day 7. Increased Tregs, less proliferative response, and impaired cytokine-secretory properties in splenocytes from I/R day 7 mice were all partially reversed by liposome clodronate injection. **$P < 0.05$ compared with I/R day 7 ($n = 5$-6).

Besides the increase in Treg population, splenocytes from tolerogenic I/R day 7 animals also showed significantly different functions following various stimuli. The proliferative response against combined anti-CD3 and anti-CD28 antibody stimulation was significantly impaired, suggesting the possible anti-proliferative effect of the pre-expanded Treg population. In addition, splenocytes from tolerogenic animals also showed an impaired cytokine response upon LPS stimulation. Despite the well-established suppressive function of Tregs in the pathogenic T-cell response, the

mechanism leading to impairment of T-cell-independent cytokine-secretory response upon LPS stimulation in this study is not certain. However, according to several reports, Tregs could also suppress acute-phase or innate immune responses via several different mechanisms.[23–25] Kinsey et al.[21] also showed that I/R injury in Treg-depleted state resulted in increased kidney neutrophil and macrophage infiltration, suggesting the role of Tregs in suppressing innate immune response *in vivo*. This impaired innate immune response of splenocytes to LPS stimulation in tolerogenic

animals could also be one other possible mechanism that contributes to protection from subsequent I/R injury wherein inflammatory responses have an important role. We also observed a marked decrease in Ly6G^{+} neutrophil infiltration in preconditioned ischemic kidneys. Among tissue cytokines, IL-6, a well-known inflammatory cytokine, also decreased significantly in preconditioned ischemic kidneys. In contrast, the levels of other cytokines, such as tumor necrosis factor-α and interferon-γ, did not show any difference between the groups.

Macrophages and DCs are all derived from monocyte/macrophage-lineage common precursor cells in bone marrow and share many surface markers, including CD11c and F4/80, and can only be discriminated by their functions, which are often very difficult to demonstrate in an *in-vivo* system. DCs are professional antigen-presenting cells linking innate and adaptive immunity and exert important functions in induction of both immunity and tolerance.[11,12,26] Among several mechanisms involved in peripheral tolerance induction by DCs, induction of Tregs by tolerogenic DCs has recently been documented.[27,28] Therefore, we hypothesized that CD11c^{+} DCs might have a crucial role in mediating tolerance induction, because we observed that ischemic preconditioning was associated with immunological tolerance that was characterized by increased spleen Tregs, and also by impaired immune response of splenocytes against mitogenic and non-mitogenic stimuli. However, because of the absence of a specific surface marker that can identify DCs, we used the CD11c^{+} population and designated them as CD11c^{+} macrophages/DCs. We first found that the percentage of peripheral blood CD11c^{+} CD11b^{+} cells that are likely to be myeloid DCs increased significantly in the tolerogenic I/R day 7 state. The Offner study also observed an increased percentage of CD11b^{+} macrophages/monocytes in peripheral blood 4 days following ischemic stroke. On the assumption that macrophage population is not present in peripheral blood, the authors raised the possibility that these CD11b^{+} cells might represent tolerogenic DCs that potentiated the activation of Tregs and simultaneously reduced the activation of effector T cells, as in our study.[14] Although an increase in these cells was not detected in the splenocytes, we performed a phenotypic analysis of spleen CD11c^{+} macrophages/DCs. A higher percentage of splenic CD11c^{+} cells expressed activation markers in I/R day 7 animals and they secreted more IL-10 and IL-6. According to classical theory, splenic CD11c^{+} cells from I/R day 7 mice are thought to be in their semi-mature stage because they secrete more IL-6 and IL-10, but not detectable IL12p70, with a relatively higher surface expression of co-stimulatory molecules. It is currently believed that tolerogenic DCs can only be defined by their function, rather than by their specific lineage. Promotion of conversion from naïve CD4^{+} T cells to Tregs and release of IL-10 are thought to be mechanisms underlying tolerogenic DCs and DCs in the semi-mature stage of maturation, as in our study, are known to induce tolerance by promoting differentiation from naïve T cells into Tregs *in vitro* and *in vivo*.[27,29–31] To examine the possible role of IL-10

in the protective effect of ischemic preconditioning, we measured IL-10 levels and also performed immunolocalization in sham day 7 or tolerogenic I/R day 7 kidney tissues. Unexpectedly, IL-10-positive cells were mainly identified in renal papilla and compared with sham kidneys, and the number of IL-10-positive cells and staining intensity increased in I/R day 7 kidneys. This parallels with the kidney IL-10 level measured by cytometric bead array, showing a significant induction of IL-10 in preconditioned kidneys. However, we could not detect any significant reduction of IL-10- or IL-10-positive cell number in liposome clodronate-treated preconditioned kidneys. Therefore, the role of IL-10 in DC–Treg interaction in ischemic preconditioning needs to be further assessed.

In this study, systemic CD11c^{+} macrophage/DC depletion induced by liposome clodronate injection resulted in partial loss of the beneficial effect of kidney ischemic preconditioning on kidney function and histology. The anti-inflammatory effect observed in preconditioned ischemic kidneys was also partially lost in these cell-depleted preconditioned kidneys. This is in contrast with a previous report demonstrating that infiltrated macrophage did not affect ischemic preconditioning.[32] However, the authors of that study used a quite different depletion strategy in that liposome clodronate was administered on day 6 after initial I/R. Given that the possible DC–Treg interaction in preconditioning might need several days, and also according to a recent paper by Kim et al.,[33] who demonstrated that phenotypic change in kidney DCs already occurred on day 3 following I/R, depletion strategy in our study seems to be more reasonable. However, more importantly, we observed that the immunophenotype alterations of splenocytes observed in tolerogenic I/R day 7 animals were also partially reversed by systemic CD11c^{+} cell depletion, further indicating that these CD11c^{+} macrophages/DCs might participate in immune cell-mediated tolerance induction in kidney ischemic preconditioning. The percentage of CD4^{+} CD25^{+} Tregs that increased in tolerogenic I/R day 7 animals decreased significantly in CD11c^{+} cell-depleted animals. In addition, less proliferative response upon TCR stimulation or impaired cytokine-secretory property upon LPS challenge, presumably due to expanded Treg population in tolerogenic splenocytes, also showed a partial reversal by systemic CD11c^{+} cell depletion. Despite several meaningful findings, controversies regarding the specificity of cell types still exist. However, because of the overlapping surface marker, using CD11c-DTR transgenic mice might also bring about the same result of nonspecific depletion of macrophages/DCs. The recent view is that macrophages are immature DCs, DCs are immature macrophages, and that conversion between these two cell types can be achieved according to environmental milieu. Liposome clodronate injection in our study did not affect other cell types, with a significant effect on systemic CD11c^{+} CD11b^{+} cells, and this depletion was associated with partial loss of ischemic preconditioning effect.

In summary, we have provided evidence that kidney I/R injury induced a strong negative signal to the peripheral immune system that is thought to be partially mediated by Tregs and/or CD11c$^+$ macrophages/DCs. Profound immunosuppression following initial I/R might contribute to a beneficial effect of kidney ischemic preconditioning. Recently, trials utilizing genetically modified tolerogenic DCs for tolerance induction are getting more attention for possible clinical application in various autoimmune diseases or organ transplantation. Identifying the mechanisms involved in peripheral tolerance induction might be an attractive way for developing various strategies in the prevention or treatment of AKI.

METHODS
Animals
Six-to-eight week-old male C57BL/6 (weight, 20–25 g) or Foxp3^{EGFP} mice (Jackson Laboratory, Bar Harbor, ME, USA) were used. They were provided standard food and water and all animal procedures were in accordance with the criteria established by the Animal Care Committee of Korea University for the care and use of laboratory animals in research.

Experimental design
We performed a well-established delayed kidney ischemic preconditioning animal model. Mice were subjected to bilateral renal pedicle clamping for 30 min or a sham operation on day 0 and were subjected to an additional 30 min of bilateral ischemia on day 7. Animals were killed on day 8 and biochemical and histological examinations were done. For another set of experiments to determine the functional significance of Tregs in ischemic preconditioning, we used depletion and adoptive transfer strategies. Anti-CD25mAb or control IgG was administered intraperitoneally on the same day of initial I/R and a second I/R injury was done on day 7. For adoptive transfer of Tregs, CD4$^+$ GFP$^+$ cells from Foxp3^{EGFP} mice were isolated and administered to Treg-depleted mice on day 3 following the initial I/R injury (sham + I/R vs I/R + I/R vs I/R + PC61 + I/R vs I/R + PC61 + Tregs + I/R).

The serum creatinine level was measured using a Hitachi 747 automatic analyzer (Black Scientific Inc., Bohemia, NY, USA) and histological kidney injury was examined in periodic acid Schiff-stained kidney tissue sections. Apoptotic cells in paraffin-embedded kidney tissue sections were identified using ApopTagPlus (Intergen, Purchase, NY, USA) and the number of apoptotic cells in the outer medulla was semi-quantitatively measured by counting 8–10 HPF ($\times$ 200) sections and compared between the groups. For assessment of tissue inflammation, the number of Ly6G-positive neutrophil-infiltrated cells in the outer medulla were counted and the tissue cytokine and chemokine levels were measured by cytometric bead array (Mouse Inflammation Kit, BD Bioscience, San Jose, CA, USA). Immunohistochemistry for IL-10 was also performed in vehicle/liposome clodronate-treated kidneys (I/R day 7 vs I/R + LC day 7) (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

In vivo trafficking of adoptively transferred Tregs
In a separate set of mice, CD4$^+$ GFP$^+$ Tregs from Foxp3^{EGFP} mice (1 $\times$ 10^5) were adoptively transferred following depletion of Tregs (I/R + Treg depletion + Tregs) on day 3 following initial I/R. On day 7 a second I/R was done and on day 8 mice were perfusion fixed with phosphate-buffered saline (PBS) and 4% paraformaldehyde, and

kidneys, lung, spleen, lymph nodes, and liver were proceeded with sequential fixation with 5% sucrose in PBS overnight at 4°C, 30% sucrose in PBS for 8 h at 4°C, and frozen at −70°C. Fluorescence microscopy was applied on 4–8-m cryosections. Cryosections of thickness 3 µm were cut, air-dried, rinsed, and then mounted using Vectashield mouting media with 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA). Indirect immuno-fluorescence microscopic images were obtained with Olympus BX51, and collected using ImagePro software (Mediacybernetics, Bethesda, MD, USA).

Liposome clodronate for depletion of CD11c$^+$ macrophages/DCs
In a separate experiment, to examine the role of CD11c$^+$ macrophages/DCs in kidney ischemic preconditioning, liposome clodronate or PBS was injected on days 1 and 3 following initial I/R. Flow cytometric detection of blood and spleen CD11b$^+$ CD11c$^+$ or CD8$^+$ CD11c$^+$ cells on day 7 following I/R was done to confirm the systemic depletion of CD11c$^+$ cells. Mice were then subjected to additional I/R injury on day 7 and killed on day 8, and all the parameters that were previously mentioned were measured again to define the role of CD11c$^+$ cells in kidney ischemic preconditioning (sham + I/R vs I/R + I/R vs I/R + liposome clodronate + I/R).

Isolation of spleen mononuclear cells (MNCs), flow cytometry, and measurement of proliferative and cytokine-secretory responses
In order to examine the mechanisms of immune cell-mediated tolerance induction in kidney ischemic preconditioning, MNCs from spleens of sham day 7 (sham day 7), tolerogenic I/R day 7 (I/R day 7), or liposome clodronate-treated I/R day 7 (I/R + liposome clodronate day 7) mice were isolated and their phenotypic characteristics were compared. For flow-cytometric analysis of Tregs, Abs to mouse CD4 and Foxp3 (eBioscience, San Diego, CA, USA) were used. For measurement of proliferative response, 5 $\times$ 10^5 splenocytes were cultured in the presence of mouse anti-CD3 and anti-CD28 Abs (BD BioCoatTM Mouse T-cell Activation Plates, BD Bioscience) for 48 h; the cells were labeled with BrdU for 18 h, the amount of BrdU incorporated was determined, and fold difference compared with that of negative control . To determine the innate immune response of splenocytes, 5 $\times$ 10^5 splenocytes were cultured for 24 h in the presence or absence of LPS (1 µg/ml, *Escherichia coli* O26:B6; Sigma Aldrich, St Louis, MO, USA) and the cytokine secretion in the supernatants was measured by cytometric bead array using a mouse inflammation kit.

Isolation, phenotyping of spleen CD11c$^+$ macrophages/DCs
In order to examine the phenotypic differences of CD11c$^+$ cells between sham-operated (sham day 7) and tolerogenic animals (I/R day 7), we isolated spleen CD11c$^+$ cells by using magnetic-assisted cell sorting. The degree of co-stimulatory molecule expression (CD80, CD86, and major histocompatibility complex II) and also cytokine secretion was measured.

Determination of kidney CD4$^+$ Foxp3$^+$ Tregs
Kidney single-cell suspension from sham, sham + I/R, or I/R + I/R mice was obtained and mononuclear cells were isolated by using Ficoll gradient. Following staining with anti-mouse CD45-PE Ab and anti-mouse CD4-FITC Ab, cells were fixed and permeabilized for intracellular Foxp3 staining and flow-cytometric detection of kidney Tregs was performed.

Statistical analysis

All data were expressed as mean ± s.e. and analyzed using Sigmastat. A Student t-test or non-parametric Mann–Whitney U-test was used to determine the statistical significance between the groups and a P-value < 0.05 was considered to be statistically significant.

DISCLOSURE

All the authors declared no competing interests.

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