IL-25 Elicits Innate Lymphoid Cells and Multipotent Progenitor Type 2 Cells That Reduce Renal Ischemic/Reperfusion Injury

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ABSTRACT

IL-25 is an important immune regulator that can promote Th2 immune response-dependent immunity, inflammation, and tissue repair in asthma, intestinal infection, and autoimmune diseases. In this study, we examined the effects of IL-25 in renal ischemic/reperfusion injury (IRI). Treating IRI mice with IL-25 significantly improved renal function and reduced renal injury. Furthermore, IL-25 treatment increased the levels of IL-4, IL-5, and IL-13 in serum and kidney and promoted induction of alternatively activated (M2) macrophages in kidney. Notably, IL-25 treatment also increased the frequency of type 2 innate lymphoid cells (ILC2s) and multipotent progenitor type 2 (MPPtype2) cells in kidney. IL-25-responsive ILC2 and MPPtype2 cells produced greater amounts of Th2 cytokines that associated with the induction of M2 macrophages and suppression of classically activated (M1) macrophages in vitro. Finally, adoptive transfer of ILC2s or MPPtype2 cells not only reduced renal functional and histologic injury in IRI mice but also induced M2 macrophages in kidney. In conclusion, our data identify a mechanism whereby IL-25-elicited ILC2 and MPPtype2 cells regulate macrophage phenotype in kidney and prevent renal IRI.


IL-25 (also known as IL-17E) is a member of the IL-17 cytokine gene family and is produced by several cell types, including T lymphocytes, mast cells, eosinophils, basophils, and epithelial cells in the lung and intestine. Administration of IL-25 to mice has been shown to induce a Th2 immune response characterized by the overproduction of IL-4, IL-5, and IL-13. IL-25 facilitates pathogenic Th2 cell responses, increases serum levels of IgE and blood eosinophilia, and enhances the recruitment of inflammatory cells in asthma and allergic inflammation. However, IL-25 is an important regulator of host defense and promotes immunity to helminth infections. Moreover, IL-25 is also required to limit chronic intestinal inflammation and experimental autoimmune encephalomyelitis through controlling Th1/Th17 cell responses.

The role of IL-25 in CKD was recently investigated and administration of IL-25 attenuated renal injury in mice with adriamycin nephropathy (AN) via inducing Th2 immune responses.

Four independent research groups recently identified previously unrecognized innate immune cell populations that were capable of contributing to...
Th2 cytokine responses in vivo. These cell populations were named natural helper cells, nuocytes, innate type 2 helper (Ih2) cells, or multipotent progenitor type 2 (MPP\textsuperscript{type2}) cells.\textsuperscript{12–15} Based on developmental, phenotypic, and functional similarities, natural helper cells, nuocytes, and Ih2 cells have been collectively categorized as group 2 or type 2 innate lymphoid cells (ILC2s).\textsuperscript{16,17} In response to the epithelial cytokines IL-25 and IL-33, ILC2s expand and produce large amounts of type 2 cytokines, particularly IL-13 and IL-5 through the expression of the receptors of these cytokines, IL-17RB and ST2, respectively. ILC2s play critical roles in promoting immunity to helminth parasites, allergic airway inflammation, and lung epithelial repair.\textsuperscript{12,14,18,19} In contrast with ILC2s, MPP\textsuperscript{type2} cells express unique cell surface markers and exhibit the ability to differentiate into cells of the monocyte and granulocyte lineages, suggesting that MPP\textsuperscript{type2} cells may be a distinct population.\textsuperscript{15,20} Administration of IL-25 promotes the accumulation of ILC2 and MPP\textsuperscript{type2} cells at multiple tissue sites, whereas whether IL-25 induces expansion of ILC2 and MPP\textsuperscript{type2} cells in kidney is unknown.

Ischemic/reperfusion injury (IRI) is the primary cause of AKI and is also relevant to a number of clinical situations, including kidney transplantation. Macrophages contribute to the initiation of IRI through secretion of cytokines, recruitment of neutrophils, and induction of epithelial cell apoptosis and also play an important role in recovery or regeneration processes from IRI by modulating immune responses against inflammation.\textsuperscript{21–23} We previously reported that IL-25 induced Th2 immune responses by increasing levels of IL-4, IL-5, and IL-13 in serum, kidney, and kidney draining lymph nodes (KLDNs), and thereby induced alternatively activated (M2) macrophages and protected against renal injury in AN.\textsuperscript{11} In this study, we evaluated IL-25’s ability to protect against renal injury in mice with IRI, and further examined possible mechanisms underlying its effect on ILC2s, MPP\textsuperscript{type2} cells, and macrophages in kidney. Here, we provide evidence that IL-25 is a critical cytokine in both promoting Th2 immune responses and preventing renal injury in murine IRI. Interestingly, ILC2 and MPP\textsuperscript{type2} cells were expanded in kidney of mice treated with IL-25, and adoptive transfer of ILC2 or MPP\textsuperscript{type2} cells attenuated renal injury in mice with IRI via the induction of M2 macrophages in kidney.

RESULTS

IL-25 Protected against Renal Injury in IRI Mice

BUN and serum creatinine were significantly increased in bilateral IRI mice compared with those of control mice and were significantly improved in bilateral IRI mice treated with IL-25 (Figure 1, A and B). In renal IRI, renal injury was characterized by tubular necrosis, tubular dilation, cast formation, and tubular cell vacuolization. Tubular injury of posts ischemic kidney was significantly increased compared with that of sham kidney and significantly reduced in IRI mice treated with IL-25 (Figure 1, C and D). Gr-1\textsuperscript{+} neutrophil infiltration in the outer medulla of postischemic kidney was significantly increased compared with that of sham kidney and significantly reduced in IRI mice treated with IL-25 (Figure 1E). However, interstitial infiltration with F4/80\textsuperscript{+} macrophages was not reduced in the outer medulla of IRI mice treated with IL-25 compared with that of control IRI mice (Figure 1F). Together, IL-25 attenuated posts ischemic renal failure and renal IRI.

IL-25 Induced Th2 Responses and Alternatively Activated Macrophages In Vivo

To define the mechanisms underlying the protective effect of IL-25 against renal injury, we examined Th2 responses in the periphery and in kidney. In IRI mice treated with IL-25, serum levels of the Th2 cytokines IL-4, IL-5, and IL-13 were significantly increased compared with those of control and IRI mice (Figure 2A). In addition, the mRNA expression of IL-4, IL-5, and IL-13 in kidney was significantly increased in IRI mice given IL-25 compared with that of control and IRI mice (Figure 2B). To further investigate the mechanisms of IL-25’s protective effects, we examined the activation status of endogenous macrophages in the kidney. Interestingly, the kidney macrophages from IRI mice treated with IL-25 had elevated mRNA expression of M2 macrophage markers, including mannose receptor (MR), arginase, FIZZ1, YM1, and IL-10 (Figure 2C). Similarly, FACS analysis demonstrated that MR expression was significantly increased in the kidney macrophages from IRI mice treated with IL-25 compared with those from control IRI mice (Figure 2D). In addition, the expression of M2 macrophage markers, such as MR and arginase, was significantly increased in macrophages isolated from spleen, liver, and lung of IRI mice treated with IL-25 (Supplemental Figure 1), indicating the influence of IL-25 in systemic immune responses rather than a specific effect on renal macrophage function. By contrast, the expression of M1 macrophage markers, including inducible nitric oxide synthase (iNOS), TNF-\alpha, IL-1\beta, IL-6, and CCL2, was significantly lower in the kidney macrophages from IRI mice treated with IL-25 than that of control IRI mice (Figure 2, E and F).

Alternatively Activated Macrophages Promoted Tubular Cell Survival In Vitro

To determine the effects of M1 and M2 macrophages on renal tubular cells in vivo, an in vitro coculture model was established to mimic the in vivo macrophage interaction with injured tubular cells. Simulated ischemic renal tubular epithelial cells (TECs) were induced by immersing the cellular monolayer in mineral oil, and were then cocultured with M0, M1, or M2 macrophages for 1–3 days. The apoptosis of ischemic TECs was significantly increased compared with control TECs, and was further enhanced by coculture with M1 macrophages, whereas coculture with M2 macrophages resulted in a reduction of apoptosis in ischemic...
TECs (Figure 3, A and B). Furthermore, the number of TECs was significantly increased after coculture with M2 macrophages for 3 days compared with that of ischemic TECs in the absence of macrophages or coculture with M0 or M1 macrophages (Figure 3C). These data indicate that M2 macrophages promoted ischemic tubular cell survival in vitro that may partially mirror the in vivo function of M2 macrophage in renal repair and regeneration.

IL-25 Elicited ILC2 and MPPtype2 In Vivo

IL-25 was recently shown to elicit previously unrecognized innate immune cells including ILC2 and MPPtype2 in multiple anatomic sites where they contribute to Th2 immune responses and regulate inflammation. To test whether IL-25 elicits similar or distinct innate cell populations in kidney of IRI mice, we used a combination of CD45, lineage (Lin) markers (CD3, CD4, CD8α, TCRβ, TCRγδ, CD19, B220, CD49b, CD11b, CD11c, FcεR1α, Gr-1, and Ter-119), ST2, IL-7Ra, c-kit, CD90, CD44, CD25, and IL-17RB. After pregregating on CD45+ cells to exclude any contaminating kidney epithelial cells, Lin– cells in kidney were analyzed for expression of surface markers that define Lin– ST2+ IL-7Ra+ ILC2 and Lin– ST2+ IL-7Ra– c-kit+ MPPtype2 in kidney (Figure 4A).12,13 Compared with control and IRI mice, IL-25–treated IRI mice exhibited significant increases in the frequencies of Lin– ST2+ IL-7Ra+ ILC2 and Lin– ST2+ IL-7Ra– c-kit+ MPPtype2 in kidney (Figure 4, B and C). In addition, Lin– ST2+ IL-7Ra+ cells in kidney expressed CD90, CD44, CD25, and IL-17RB, a phenotype consistent with ILC2.12,13 Lin– ST2+ IL-7Ra+ c-kit+ cells in kidney expressed IL-17RB but lacked expression of CD90, CD44, and CD25, a phenotype consistent with that of MPPtype2 cells but distinct from ILC2s.15,20 Moreover, IL-25–elicited ILC2 and MPPtype2 cells were observed in multiple anatomic sites including the blood, peritoneal cavity, KLDNs, spleen, liver, and lung, which is consistent with previous studies (Figure 4E). Therefore, these data indicate that IL-25 elicits both ILC2 and MPPtype2 response in kidney, which may be associated with the induction by IL-25 of Th2 responses in kidney.

ILC2 and MPPtype2 Induced M2 Macrophages and Suppressed M1 Macrophages In Vitro

ILC2 and MPPtype2 were isolated from BALB/c mice treated with IL-25. ILC2s produced greater amounts of IL-5 and IL-13, but low amounts of IL-4, in culture supernatant with IL-2/IL-7/IL-25 stimulation (Figure 5A).12,24 By contrast, after

Figure 1. IL-25 protects against renal injury in IRI mice. (A) BALB/c mice are administered with mouse recombinant IL-25 daily for 5 consecutive days before unilateral or bilateral IRI operation. Mice are euthanized at day 1 after IRI. (B) BUN and creatinine levels are assessed in control, IRI+vehicle, and IRI+IL-25 groups at day 1 after bilateral IRI. (C) PAS-stained sections of kidney outer medulla from unilateral IRI mice treated with PBS or IL-25. (D) Semiquantitative assessment of tubular injury in the IRI+vehicle and IRI+IL-25 groups at day 1 after unilateral IRI. (E and F) Numbers of Gr1+ neutrophils and F4/80+ macrophages are assessed by immunofluorescence staining in outer medulla of kidney. Values represent the mean±SEM of evaluations from each group (n=6 per group). *p<0.05 versus IRI+vehicle. PAS, periodic acid–Schiff. Original magnification, ×200 in C.
culture with IL-3/stem cell factor/IL-25, MPP\textsuperscript{type2} produced elevated levels of IL-4 but little IL-13 (Figure 5B).\textsuperscript{15,20} To test whether ILC2 and MPP\textsuperscript{type2} regulate macrophage activation, bone marrow–derived macrophages were cultured with activated ILC2 and MPP\textsuperscript{type2} in vitro. Both ILC2 and MPP\textsuperscript{type2} increased the mRNA expression of M2 macrophage markers, including MR, arginase, FIZZ1, YM1, and IL-10. IL-10 levels were significantly increased in the supernatant of macrophages cocultured with ILC2 or MPP\textsuperscript{type2} in comparison to macrophages alone. Critically, induction of the M2 phenotype of macrophages by ILC2 and MPP\textsuperscript{type2} was blocked by IL-4/IL-13 neutralizing antibodies (Figure 5, C and D). By contrast, M1 macrophages
cocultured with ILC2 or MPPtype2 significantly reduced their proinflammatory phenotype, including iNOS, TNF-α, IL-1β, IL-6, and CCL2. TNF-α, IL-1β, and IL-6 levels were significantly decreased in the supernatant of M1 macrophages cocultured with ILC2 or MPPtype2 compared with M1 macrophages alone. The effect on M1 macrophages of ILC2 or MPPtype2 was blocked by IL-4/IL-13 neutralizing antibodies (Figure 5, E and F). Thus, these data suggest that ILC2 and MPPtype2 induce M2 macrophages and suppress M1 macrophages through producing Th2 cytokines in vitro.

Adoptive Transfer of ILC2 and MPPtype2 Attenuated Renal Injury in IRI
We next investigated the in vivo function of ILC2 and MPPtype2 by adoptive transfer study. The ILC2 and MPPtype2 were separated from BALB/c mice treated with IL-25, and adoptively transferred into IRI BALB/c mice 1 day before IRI (Figure 6A). Transfused ILC2, MPPtype2, or both ILC2 and MPPtype2 cells significantly improved renal function in bilateral IRI mice, including reduction of BUN and serum creatinine (Figure 6B). Examination of renal histology (Figure 6C) and tubular injury scoring (Figure 6D) 1 day after IRI confirmed that tubule damage was improved in IRI mice treated with ILC2, MPPtype2, or both ILC2 and MPPtype2 cells compared with that of control IRI mice. Gr-1+ neutrophil infiltration in the outer medulla of postischemic kidney was significantly increased compared with that of sham kidney and was significantly reduced in IRI mice treated with ILC2, MPPtype2, or both ILC2 and MPPtype2 cells (Figure 6E). However, interstitial infiltration with F4/80+ macrophages was not reduced in outer medulla of IRI mice treated with ILC2, MPPtype2, or both ILC2 and MPPtype2 cells compared with that of control IRI mice (Figure 6F). These data indicate that both ILC2 and MPPtype2 have protective effects on renal function and injury in mice with renal IRI.

Adoptive Transfer of ILC2 and MPPtype2 Induced Th2 Response and Alternatively Activated Macrophages in Kidney
To further investigate the mechanisms of the protective effects of ILC2 and MPPtype2 in IRI mice, we first examined whether ILC2 and MPPtype2 cells distribute into kidney. As expected, fluorescently labeled ILC2 and MPPtype2 cells were observed in...
IL-25 elicits ILC2 and MPP\textsuperscript{type2} in kidney. BALB/c mice are administered mouse recombinant IL-25 daily for 5 consecutive days before IRI operation. Indicated tissues are harvested at day 1 after IRI, and the frequency of ILC2 and MPP\textsuperscript{type2} cells are assessed by flow cytometry. (A) Representative FACS analysis showing the gating strategy to identify ST2\textsuperscript{IL-7R\alpha} ILC2 and ST2\textsuperscript{IL-7R\alpha}\textsuperscript{c-kit} MPP\textsuperscript{type2} in the CD45\textsuperscript{Lin} cells from kidney of control, IRI+vehicle, or IRI+IL-25 mice. (B and C) Frequency of ST2\textsuperscript{IL-7R\alpha} ILC2 and ST2\textsuperscript{IL-7R\alpha}\textsuperscript{c-kit} MPP\textsuperscript{type2} in the CD45\textsuperscript{Lin} cell compartment from the kidneys of control, IRI+vehicle, or IRI+IL-25 mice. (D)
both sham and IRI kidney, whereas the number of ILC2 and MPP\textsuperscript{type2} cells in IRI kidney was significantly higher than that in sham kidney (Supplemental Figure 2, A and B). In addition, fluorescently labeled ILC2 and MPP\textsuperscript{type2} cells were also observed in spleen, liver, and lung (Supplemental Figure 2C). Next, we assessed Th2 cytokine expression in kidney and phenotype of endogenous kidney macrophages. The mRNA expression of IL-4, IL-5, and IL-13 in kidney was significantly increased in IRI mice given ILC2, MPP\textsuperscript{type2}, or both ILC2 and MPP\textsuperscript{type2} cells compared with control and IRI mice (Figure 7A). Meanwhile, the endogenous kidney macrophages of IRI mice treated with ILC2 or MPP\textsuperscript{type2} expressed high levels of MR, arginase, and FIZZ1, but not YM1 and IL-10 (Figure 7, B and C). In addition, adoptive transfer of both ILC2 and MPP\textsuperscript{type2} cells elevated the expression of M2 macrophage markers in endogenous kidney macrophages, including MR, arginase, FIZZ1, YM1, and IL-10 (Figure 7, B and C). These data suggest that ILC2 and MPP\textsuperscript{type2} prevent IRI through inducing Th2 response and M2 macrophages in kidney.

**DISCUSSION**

In this study, IL-25 induced a Th2 immune response and significantly prevented renal structural and functional injury in mice with IRI. The mechanisms underlying the protective effect of IL-25 might be associated with its initiation of Th2 immune responses through expansion of innate immune cells, ILC2 and MPP\textsuperscript{type2}. IL-25–responsive ILC2 and MPP\textsuperscript{type2} cells produced a great amount of Th2 cytokines and were shown to induce M2 macrophages and suppress activation of M1 macrophages in vitro. Furthermore, adoptive transfer of ILC2 or MPP\textsuperscript{type2} cells prevented renal injury in mice with IRI via activation of Th2 response and induction of M2 macrophages in kidney, which reduced renal inflammation and promoted tissue repair. These data demonstrate that IL-25 may be a useful therapeutic strategy for AKI.

IL-25 has been shown to promote Th2 immune responses, thereby inducing allergic airway inflammation.\textsuperscript{1,5} However, IL-25 also plays a critical role in suppressing intestinal chronic inflammation, regulating adipose tissue inflammation and protecting against LPS-induced lethal endotoxemia.\textsuperscript{3,25,26} We have demonstrated that IL-25 attenuated renal injury in AN, a model of CKD, whereas the potential role of IL-25 in acute kidney disease is unclear. AKI induced by IRI is associated with an acute reduction of blood flow that produces hypoxia-induced vascular and tubular dysfunction. Innate immunity appears to be the dominant pathway of renal injury induction and progression in this model.\textsuperscript{27} Administration of IL-25 before IRI significantly improved renal function and reduced renal injury in IRI mice. This is the first report to show that IL-25 can prevent renal injury in acute kidney disease. IL-25 induced Th2 immune response in both the periphery and within the kidneys of IRI mice characterized by the overproduction of IL-4, IL-5, and IL-13, which is consistent with previous studies in intestinal chronic inflammation and autoimmune disease.\textsuperscript{3,10} Enhancing Th2 immunity with exogenous IL-4 or IL-13 was also shown to reduced renal inflammation and injury in experimental immune-mediated GN.\textsuperscript{28–31} By contrast, IL-4–deficient or STAT6–deficient mice had markedly worse renal functional and tubular injury after ischemia compared with the wild type.\textsuperscript{32} IL-4 neutralizing antibody reversed the protective effect of Sphingosine 1-phosphate 3–deficient dendritic cells in IRI mice.\textsuperscript{33} Moreover, the kidney M2 macrophages were significantly increased in IRI mice treated with IL-25. Our group first reported that IL-25 induced M2 macrophages within the kidney of AN mice and discovered that induced M2 macrophages may be an important mechanism underlying IL-25’s protective role in AN.\textsuperscript{11} Hams et al.\textsuperscript{34} recently reported that treating obese mice with IL-25 induced weight loss and improved glucose tolerance, and was associated with increased infiltration of M2 macrophages into the visceral adipose tissue (VAT).\textsuperscript{26} Similarly, Yang and colleagues demonstrated that IL-25–responsive M2 macrophages play an important role in the induction of type 2 immunity to *Heligmosomoides bakeri* infection.\textsuperscript{34} M2 macrophages, which regulate inflammation and promote tissue repair, have been applied as a treatment of experimental kidney diseases.\textsuperscript{22,35–38} For instance, Lee et al.\textsuperscript{39} found that adoptive transfer of M2 macrophages early after injury reduced renal injury in mice with IRI.\textsuperscript{22} We found that M2 macrophages protected against apoptosis of ischemic TECs and promoted proliferation of ischemic TECs in vitro. Therefore, the mechanisms underlying the renoprotective effects of IL-25 could involve its initiation of Th2 immune responses and induction of M2 macrophages.

A most interesting finding in this study was the induction by IL-25 of ILC2 and MPP\textsuperscript{type2} in kidney, demonstrated here for the first time. Treating IRI mice with IL-25 increased the frequency of ILC2 and MPP\textsuperscript{type2} cells in multiple tissue sites, including the blood, peritoneal cavity, spleen, liver, and lung, which is consistent with previous studies.\textsuperscript{15,20} Moreover, IL-25–elicited ILC2 and MPP\textsuperscript{type2} cells were observed in kidney and KDLN of IRI mice. ILC2 and MPP\textsuperscript{type2} cells have been shown to contribute to Th2 cytokine responses in helminth infections and allergic airway inflammation. Thus, it is likely that ILC2 and MPP\textsuperscript{type2} cells elicited by IL-25 are involved in the induction of Th2 response in the local environment of the kidney.

Next, we examined whether ILC2 and MPP\textsuperscript{type2} cells could regulate activation status of macrophage in vitro and in vivo. As...
Figure 5. ILC2 and MPP\textsuperscript{type2} induce M2 macrophages and suppress M1 macrophages in vitro. ILC2 and MPP\textsuperscript{type2} are isolated from BALB/c mice treated with IL-25 by flow sorting. (A and B) Sorted ILC2s are cultured with medium only or with IL-2 (10 ng/ml), IL-7 (10 ng/ml), and IL-25 (100 ng/ml) for 2 days. MPP\textsuperscript{type2} cells are incubated with medium only or with IL-3 (10 ng/ml), SCF (50 ng/ml), and IL-25 (100 ng/ml) for 2 days. IL-4, IL-5, and IL-13 levels in culture supernatants are measured by ELISA. Data represent the mean ± SEM of four independent experiments. (C and D) Bone marrow macrophages are incubated with complete medium (M0), recombinant IL-4/IL-13 (M2), or ILC2 or MPP\textsuperscript{type2} in the presence of IL-4/IL-13 neutralizing antibodies or control rat IgG for 24 hours. The mRNA expression of MR, arginase, FIZZ1, YM1, and IL-10 is assessed by real-time PCR in ILC2s, MPP\textsuperscript{type2} cells, and bone marrow macrophages. The levels of IL-10 in culture supernatants are measured by ELISA. Data represent the mean ± SEM of five independent experiments. *P < 0.05 versus M0;
we previously demonstrated, IL-25 treatment reduced renal injury of AN via induction of Th2 response and M2 macrophages in vivo. However, IL-25 did not promote induction of M2 macrophage directly and IL-25-modulated Th2 cells induced M2 macrophages in vitro. Here, we found that IL-25–responsive ILC2 and MPP<sub>type2</sub> cells produced greater amounts of Th2 cytokines and induced M2 macrophages in vitro. Moreover, IL-25–responsive ILC2 and MPP<sub>type2</sub> cells are able to suppress LPS-activated macrophages, leading to reduced proinflammatory mediator production in vitro. However, the effects on macrophages of ILC2 and MPP<sub>type2</sub> cells were dependent on their secretion of Th2 cytokines. Molofsky et al. reported that ILC2s are the major source of IL-5 and IL-13 in VAT and deletion of ILC2s causes significant reduction of M2 macrophages in VAT. To determine whether ILC2 and MPP<sub>type2</sub> cells could induce M2 macrophages in kidney, the effect of ILC2 and MPP<sub>type2</sub> cells was further examined in IRI mice. Indeed, administration of ILC2 or MPP<sub>type2</sub> cells not only attenuated renal functional and histologic injury in IRI mice but also induced M2 macrophages in kidney. Therefore, this study has discovered a new mechanism underlying the renoprotective effects of IL-25—that IL-25–responsive ILC2 and MPP<sub>type2</sub> cells directly modulate macrophage phenotype, thereby contributing to recovery of renal injury in IRI mice. In addition to the effects of IL-25 on T cells, macrophages, ILC2s, and MPP<sub>type2</sub> cells, IL-25 may act on other immune cells including mast cells, natural killer cells, and dendritic cells, which play a critical role in progression of acute kidney disease. However, the IL-25 receptor is also expressed on renal TECs, so whether IL-25 regulates function of TECs needs further investigation.

The interaction between the innate immune system and renal injury is a prominent area of research because of the current progress of innate immune studies in kidney disease. Although previous studies from our group and others have outlined the importance of M2 macrophages in reducing renal inflammation and promoting tissue repair, this study suggests a future potential role for modulating cytokine activity in kidney disease, either to upregulate IL-25 or other Th2 cytokines directly, thereby artificially promoting ILC2 and MPP<sub>type2</sub> expansion and localized M2 macrophage polarization. This study raises the potential for IL-25 and IL-25–elicited innate immune cells as therapeutics for attenuating tubular injury of postischemic kidney.

**CONCISE METHODS**

**IRI Murine Model and IL-25 Administration**

Six- to 8-week-old male BALB/c mice obtained from the Shanghai Laboratory Animal Center of Chinese Academy of Sciences were used in this study. The Animal Ethics Committee of Xinxiang Medical University approved all procedures. Unilateral or bilateral renal ischemia was imposed in BALB/c mice under isoflurane anesthesia. The kidneys were exposed via a midline abdominal excision; the left renal pedicle or both renal pedicles was clamped for 30 minutes using a nontraumatic sterile clamp (Roboz). After clamp removal, the kidney was inspected for restoration of blood flow as evidenced by returning to its original color before closing the wound with standard sutures. After the procedure, mice were given 1 ml of warm normal saline intraperitoneally to prevent dehydration. The animals were kept at a constant temperature (37°C) during the procedure and allowed to recover. Animals subjected to sham operation were used as controls.

BALB/c mice were divided into three groups: control, IRI with vehicle, and IRI with IL-25 treatment. For IL-25 treatment, mice were administered 0.5 µg mouse recombinant IL-25 (R&D Systems) intraperitoneally daily for 5 consecutive days before IRI operation. The dose and duration were selected according to previously published studies. Control animals received PBS only. All mice were euthanized at day 1 after IRI operation. Blood, peritoneal exudate cells, KDLN, spleen, liver, lung, and kidney were harvested for analysis. ILC2 and MPP<sub>type2</sub> cells were examined in multiple tissues by flow cytometry. For functional assessment, mice were injected with IL-25 or vehicle (PBS) intraperitoneally daily for 5 consecutive days, and then underwent bilateral ischemia-reperfusion for 30 minutes on both kidneys by midline abdominal incision. BUN and creatinine levels were measured using a Hitachi 747 automatic analyzer.

**ILC2 and MPP<sub>type2</sub> Cell Labeling and Adoptive Transfer to IRI Mice**

BALB/c mice were divided into five groups: control, IRI with vehicle, IRI with ILC2 treatment, IRI with MPP<sub>type2</sub> treatment, and IRI with IL-25 treatment. For ILC2 treatment, mice were administered 3 x 10<sup>5</sup> ILC2, 3 x 10<sup>5</sup> MPP<sub>type2</sub> cells, 1 x 10<sup>5</sup> ILC2, and 1 x 10<sup>5</sup> MPP<sub>type2</sub> cells intraperitoneally daily for 5 consecutive days before IRI operation. Unilateral or bilateral renal ischemia-reperfusion was initiated 1 day before IRI operation. All mice were euthanized at day 1 after IRI operation. Blood, peritoneal exudate cells, KDLN, spleen, liver, lung, and kidney were harvested for analysis. The distribution of CFSE-labeled ILC2 and MPP<sub>type2</sub> was analyzed in kidney, spleen, liver, and lung sections by inverted fluorescence microscopy. The number of transfused ILC2 and MPP<sub>type2</sub> was quantitated in 8 independent experiments. SCF, stem cell factor.

**P<0.05 versus rat IgG. (E and F)** Bone marrow macrophages are stimulated with LPS (M1) for 24 hours, and then cocultured with ILC2 or MPP<sub>type2</sub> in the presence of IL-4/IL-13 neutralizing antibodies or control rat IgG for 24 hours. The mRNA expression of iNOS, TNF-α, IL-1β, IL-6, and CCL2 is assessed by real-time PCR in ILC2s, MPP<sub>type2</sub> cells, and bone marrow macrophages. The levels of TNF-α, IL-1β, and IL-6 in culture supernatants are measured by ELISA. Data represent the mean ± SEM of five independent experiments. SCF, stem cell factor.

**P<0.05 versus M1; aP<0.05 versus rat IgG.**
Cell Suspension Preparation

Spleen and KDLNs were isolated, minced, and digested for 30 minutes at 37°C in RPMI 1640 medium containing 1 mg/ml collagenase D (Roche) and 100 μg/ml DNase I (Roche). The digested cell suspension was then passed through a 40-μm cell strainer. Kidney, liver, and lung were perfused with saline before removal and digested with collagenase and DNase as previously described. Kidney, liver, and lung were cut into 1- to 2-mm³ pieces, placed in DMEM containing 1 mg/ml collagenase IV (Sigma-Aldrich), and 100 μg/ml DNase I (Roche) for 40 minutes at 37°C with intermittent agitation. The digested cell suspension was then passed through 40-μm cell strainer.

The cell suspensions were allowed to settle for 10 minutes, after which the upper three-fourths (lower density cells) was removed for subsequent assays. F4/80+ macrophages were sorted from kidney, spleen, liver, and lung by FACS. Sorted macrophages were used for real-time PCR analyses to detect phenotypes of these macrophages. Some sorted macrophages were stained with anti-mouse mannose receptor (BioLegend) or iNOS (BD Biosciences) and analyzed by flow cytometry.

FACS Analyses

For FACS analysis, single-cell suspensions were stained with Fc block/anti-CD16/32 (2.4G2) and antibodies to CD45.2 (104), ST2 (RMST2-2), IL-7Rα (A7R34), c-kit (2B8), CD90.2 (30-H12), CD44 (IM7), CD25 (PC61), and IL-17RB (9B10), as well as antibodies to the following T cell, B cell, natural killer cell, monocyte/macrophage, dendritic cell, eosinophil, neutrophil, and erythroid cell lineages (referred hereafter as Lin): CD3 (145-2C11), CD4 (GK1.5), CD8α (53-6.7), TCRβ (H57-597), TCRγδ (eBioGL3), CD19 (1D3), B220 (RA3-6B2), CD49b (DX5), CD11b (M1/70), C11c (N418), FcεRIα (MAR-1).
Gr-1 (RB6-8C5), and Ter-119 (all purchased from eBioscience or BioLegend). Cells were analyzed on an LSR II flow cytometer (BD Biosciences). For FACS sorting, single-cell suspensions were pre-gated on hematopoietic cells using anti-CD45.2 antibody, and then lineage (CD3/CD4/CD8α/TCRβ/CD19/B220/CD49b/CD11b/CD11c/FcγRIa/Gr-1/Ter-119) was used to exclude immune cells and 4′,6-diamidino-2-phenylindole was used to exclude dead cells. ILC2 and MPPtype2 cells (Lin^− ST2^+ IL-7Rα^+ c-kit^+) were sorted using a FACSAria II (BD Biosciences). After sorting, cells were used for phenotypic and functional assays.

**ILC2 and MPPtype2 Coculture with Macrophages**

ILC2 and MPPtype2 were isolated from BALB/c mice treated with IL-25. ILC2s were cultured in RPMI 1640 medium, supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml), plus IL-2 (10 ng/ml), IL-7 (10 ng/ml), and IL-25 (100 ng/ml) for 2 days. MPPtype2 cells were incubated in the presence of IL-3 (10 ng/ml), stem cell factor (50 ng/ml), and IL-25 (100 ng/ml) for 2 days. Cell-free supernatants were assessed for IL-4, IL-5, and IL-13 cytokine production by ELISA (eBioscience). Primary cultures of murine macrophages were obtained from bone marrow of BALB/c mice by a previously described technique.11-14 Macrophages derived from bone marrow were cultured in RPMI 1640 medium, supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml), plus 10 ng/ml macrophage colony-stimulating factor for 6 days, and F4/80+ macrophages were sorted by flow cytometry. Cell viability was >95%. Macrophages were seeded onto 12-well culture plates (2×10^5 cells/well) and incubated with complete medium (referred to as M0), mouse recombinant IL-4/IL-13 (10 ng/ml each; R&D Systems) (referred to as M2), ILC2 (2×10^4 cells/well) or MPPtype2 (2×10^4 cells/well) in the presence of IL-4/IL-13 neutralizing antibodies (eBioscience), or control rat IgG1 (eBioscience) for 24 hours. In parallel, macrophages (2×10^5 cells/well) were stimulated with LPS (100 ng/ml; Sigma-Aldrich) (referred to as M1) for 24 hours, and then cocultured with ILC2 (2×10^4 cells/well) or MPPtype2 (2×10^4 cells/well) in the presence of IL-4/IL-13 neutralizing antibodies (eBioscience) or control rat IgG1 (eBioscience) for 24 hours. Macrophages were then used for real-time PCR and ELISA analyses to detect macrophage phenotype.

**Primary Culture of Mouse Renal TECs**

Primary mouse TECs were generated following established methods adapted from Doctor et al.41 In brief, kidneys were harvested after cardiac perfusion with saline to remove blood cells. The tissue from the outer cortex was cut into pieces of approximately 1 mm^3 and then digested in DMEM containing 1 mg/ml collagenase IV (Sigma-Aldrich), and 100 μg/ml DNase I (Roche) for 40 minutes at 37°C with intermittent agitation. Renal TECs were separated by centrifugation using Percoll solution and cultured in defined K1 medium: DMEM/F12 medium supplemented with 10 ng/ml EGF, 1 ng/ml PGE1, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite, 5×10^-11 M triiodothyronine, 5×10^-8 M hydrocortisone, 100 U/ml penicillin, 100 μg/ml streptomycin, 25 mM HEPES, and 5% FBS.

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Figure 7. Adoptive transfer of ILC2 and MPPtype2 induces Th2 response and alternatively activated macrophages in kidney. (A) mRNA expression of IL-4, IL-5, and IL-13 in kidney from control, IRI+vehicle, IRI+ILC2, IRI+MPPtype2, and IRI+ILC2 and MPPtype2 groups is examined by real-time PCR, and is expressed relative to the control of each experiment. F4/80+ kidney macrophages are sorted by flow cytometry in control, IRI+vehicle, IRI+ILC2, IRI+MPPtype2, and IRI+ILC2 and MPPtype2 groups at day 1 after IRI. (B) The mRNA expression of MR, arginase, FIZZ1, YM1, and IL-10 is quantified by real-time PCR in F4/80+ kidney macrophages. (C) The expression of MR (mean fluorescence intensity) in F4/80+ kidney macrophages is assessed by flow cytometry. Values represent the mean±SEM of evaluations from each group (n=6 per group). *P<0.05 versus IRI+vehicle.

**BASIC RESEARCH**
Simulated Ischemic Renal TECs Coculture with Macrophages

Ischemia in renal TECs was simulated by immersing the cellular monolayer in mineral oil according to the protocol of Meldrum et al. This immersion simulated ischemia by restricting cellular exposure to oxygen and nutrients as well as by limiting metabolite washout. Briefly, renal TECs (1×10^6) were placed in six-well tissue culture plates in serum-free K1 medium for 24 hours, washed twice with PBS, and immersed in mineral oil for 60 minutes at 37°C. After extensive washing with PBS, TECs were incubated in K1 medium. Bone marrow–derived macrophages were polarized to M0 (medium), M1 (LPS), or M2 (IL-4/IL-13) in vitro. The M0, M1, or M2 macrophages were seeded on a 0.4-μm Transwell insert (Falcon) and cocultured with ischemic renal TECs for 1–3 days. TECs were exposed to serum-free K1 medium alone as the non-ischemic control. Numbers of renal TECs were determined at each time point by trypsinizing and counting the cells. Apoptosis of TECs at 1 day after the coculture were measured using FACS with 7-aminoactinomycin D and Annexin V staining according to the manufacturer’s protocol (BD Biosciences).

ELISA of Cytokines

IL-4, IL-5, IL-13, IL-10, TNF-α, IL-1β, and IL-6 levels in sera and culture supernatants were assayed using an ELISA kit purchased from eBioscience. ELISA was performed according to the manufacturer’s protocol.

Quantitative RT-PCR

F4/80+ endogenous renal macrophages were sorted by FACS. Cell viability was >95%. Total RNA was isolated from kidney or endogenous renal macrophages by the RNeasy Mini Kit (Qiagen), and was reverse-transcribed with the First-Strand cDNA Synthesis Kit (Fermantas). Real-time PCR was performed on the Rotogene-6000 Real-Time Thermal Cycler (Corbett Research) using the SYBR Master Mix (Invitrogen). The analysis method was as previously described and the PCR primer sequences are presented in Supplemental Table 1.

Histology and Immunofluorescence

Coronal sections of kidney tissue were stained with periodic acid–Schiff. Histologic changes in the corticomedullary junction and outer medulla were evaluated semiquantitatively. Briefly, tubular damage was estimated in 8–10 high-power fields (×200) per section by using a scoring system based on the percentage of damaged tubules per field (0, normal; 1, <10%; 2, 10%–25%; 3, 25%–50%; 4, 50%–75%; and 5, >75%). The mean score of each animal was compared. To avoid selection bias, the areas to be viewed for morphometric analysis were anatomically identical for each section and positioned before microscopic visualization.

For immunofluorescence staining, rat anti-mouse Gr-1 (Ly-6G, 1/100; BioLegend) or F4/80 (1/100; eBioscience) was used as the primary antibody and AF488 goat anti-rat IgG (1/1000; Invitrogen) as the secondary antibody. Control rat IgG to primary antibodies was included in staining. The number of interstitial Gr-1+ and F4/80+ cells was quantitated in 8–10 nonoverlapping outer medulla fields (×400).

Statistical Analyses

Renal functional data were log-transformed before analysis to stabilize the variance. Statistical tests included the unpaired, two-tailed t test using Welch’s correction for unequal variances and one-way ANOVA with Tukey’s multiple comparison test. Statistical analyses were performed using Prism software (version 5; GraphPad). Results are expressed as the mean±SEM. P<0.05 was considered statistically significant.

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DISCLOSURES

None.

REFERENCES


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