Contrasting roles for STAT4 and STAT6 signal transduction pathways in murine renal ischemia-reperfusion injury

Naoko Yokota,1 Melissa Burne-Taney,1 Lorraine Racusen,2 and Hamid Rabb1

1Division of Nephrology, 2Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

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Yokota, Naoko, Melissa Burne-Taney, Lorraine Racusen, and Hamid Rabb. Contrasting roles for STAT4 and STAT6 signal transduction pathways in murine renal ischemia-reperfusion injury. Am J Physiol Renal Physiol 285: F319–F325, 2003. First published April 22, 2003; 10.1152/ajprenal.00432.2002.—Recent data support a modulatory role for CD4 T cells in experimental renal ischemia-reperfusion injury (IRI). CD4 T cells can functionally differentiate to either a Th1 (IFN-γ producing) or the counterbalancing Th2 (IL-4) phenotype. The enzymes signal transducers and activators of transcription (STAT) 4 and STAT6 regulate Th1 or Th2 differentiation and cytokine production, respectively. We therefore hypothesized that mice that were STAT4 deficient would be protected from renal IRI and that STAT6-deficient mice would have a more severe course. Intracellular cytokine staining of splenocytes from STAT4−/− or STAT6−/− exhibited distinct IFN-γ and IL-4 cytokine expression profiles. STAT6−/− had markedly worse renal function and tubular injury postischemia compared with wild type. STAT4−/− had only mildly improved function. Renal phagocyte infiltration and ICAM-1 upregulation were similar in STAT4−/−, STAT6−/−, and wild type. To evaluate if the mechanism of the marked worsening in the STAT6−/− mice could be due to IL-4 deficiency, IL-4-deficient mice were studied and had similar postischemic phenotype to STAT6−/− mice. These data demonstrate that the STAT6 pathway has a major protective role in renal IRI. IL-4 deficiency is a likely mechanism underlying the STAT6 effect. A “yin-yang” role for inflammation is emerging in renal IRI, similar to recent observations in atherosclerosis.

Th1/Th2 cells; inflammation; interleukin-4

ISCHEMIA-REPERFUSION INJURY (IRI) is the main cause of intrinsic acute renal failure (ARF). Native kidney ARF is associated with a greater than 30% mortality (22, 24), and transplant ischemic ARF adversely affects both short- and long-term allograft function (25). Inflammation is currently established to be an important pathogenic component in the development of renal IRI, but the cellular basis is incompletely understood. Although the neutrophil was originally focused on as the major inflammatory mediator of renal IRI, recent data support an important role for the T cell. Mononuclear leukocytes are found in the vasa recta in biopsies of patients with ischemic ARF (21). CTLA-4Ig, which blocks T cell costimulation via the CD28-B7 pathway, improved cold renal IRI in a rat model (23). Furthermore, treatment with a monoclonal antibody specific to B7 molecule has also been found to be protective in a rat model of renal IRI (4). Targeted gene knockout mice have enabled more detailed mechanistic evaluation of T cell pathways in disease. CD4 and CD8 double-knockout mice are significantly protected from renal IRI (18). Nu/nu mice, which are T cell deficient, are also protected from renal injury postischemia and the injury phenotype is restored after adoptive transfer of naïve T cells into nu/nu mice (2). Furthermore, CD4 knockout mice are also significantly protected from renal IRI, whereas CD8 knockout mice are not. Adoptive transfer of naïve CD4 T cells into CD4 knockout mice resulted in reconstitution of the injury level (2). IFN-γ and CD28 pathways were shown to play an important role in T cell-mediated injury in this study. Mice depleted of T cells, particularly CD4 cells, using monoclonal antibodies, are also afforded significant protection from renal dysfunction postischemia (28).

CD4 T cells functionally differentiate into two different phenotypes, Th1 and Th2 cells (1). Differentiation into Th1 and Th2 cells is characterized by specific cytokine expression. IL-12 is required for Th1 cell differentiation, and this is followed by the production of IFN-γ, whereas Th2 cells require IL-4 production followed by IL-4, IL-5, IL-6, IL-10, and IL-13 secretion (1, 27). Given recent data supporting a role for T cells, particularly the CD4+ T cell in renal IRI, we hypothesized that the Th1 inflammatory pattern is pathogenic in renal IRI, whereas the Th2 pattern is protective (opposite to what has been proposed in asthma) (26). Recent availability of gene knockout mice for signal transducers and activators of transcription (STAT) 4, which mediate Th1 differentiation, or STAT6, which is required for Th2 differentiation, allowed us to investigate this question (9, 10). We evaluated mice deficient in STAT4 and STAT6 and compared their cytokine responses and outcomes in experimental renal IRI. We found that the T cells from STAT4−/− mice had a blunted IFN-γ response to stimulation, and those from STAT6−/− mice had diminished IL-4. The STAT6−/− mice had a marked worsening of kidney injury follow-

Address for reprint requests and other correspondence: H. Rabb, Div. of Nephrology, Johns Hopkins Univ. School of Medicine, Rm. 970, 720 Rutland Ave., Baltimore, MD 21205 (E-mail: hrabb1@jhmi.edu).

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Fig. 1. Cytokine characteristics of signal transducers and activators of transcription (STAT)-deficient animals. T cells from STAT4−/− mice demonstrated a reduced production of IFN-γ and vigorous production of IL-4 production. In contrast, T cells from STAT6−/− mice had an opposite production of cytokine pattern with active IFN-γ production and minimum IL-4 production.

METHODS

Mice. STAT4−/− (C.129S2-Stat4tm1Gru), STAT6−/− (C.129S2-Stat6tm1Gru), IL-4−/− (Balb/c-IL-4tm2Nnt), and IFN-γ−/− [C.129S7(B6)-Ifngtm1Gru] were purchased from Jackson Laboratory (Bar Harbor, ME). Background strain-, age-, and sex-matched control mice were used. Mice were held under pathogen-free conditions according to National Institutes of Health guidelines.

Cell characterization. Intracellular cytokine staining (ICCS) was used to characterize the cells obtained from STAT-deficient mice. An aliquot of T cell suspension from spleen was stimulated with anti-CD3 antibody (CD3ε chain, 145–2C11, BD Pharmingen, San Diego, CA) in the presence of Brefeldin A (GolgiPlug, BD Pharmingen) in RPMI containing 10% fetal bovine serum. After 12 h of stimulation, cells were harvested, treated with anti-mouse CD16/CD32, and stained with pan-T cell surface marker CD90.2 (Thy-1.2, 30-H12, BD Pharmingen). Cells were fixed with 1% formaldehyde (Cytofix/Cytoperm, BD Pharmingen) and stained with a second antibody specific to an intracellular cytokine, such as IFN-γ (XMG1.2, BD Pharmingen) and IL-4 (11B11, BD Pharmingen), under continuous saponin permeabilization (Perm/Wash, BD Pharmingen), and then analyzed.

Renal IRI. Mice were anesthetized using a ketamine-xylazine mixture (150 μg/g ketamine, 3 μg/g xylazine), and an incision was made on the central abdomen. Avoiding intestines and bowl, microvascular clamps were applied to bilateral renal pedicles. After 35 min of renal ischemia, clamps were removed and the incision was closed. During the procedure, mice were well hydrated and their body temperature was controlled at ~37°C using an adjustable heating pad. Animals were kept under veterinarian observation postischemia.

Analysis of renal function. Serum creatinine was used for the evaluation of renal function postischemia. Previous work validated serum creatinine as a direct measure of kidney function up to 72 h postischemia (16). At 0, 24, 48, and 72 h postischemia, mice were bled from their tail vein using heparinized tubes. Serum samples were analyzed on a COBAS Mira chemical analyzer (Roche, Basel, Switzerland), using creatinine reagent 557 (Sigma, St. Louis, MO).

Histological and immunohistochemical analysis. Formaldehyde-fixed paraffin sections of kidney were stained with hematoxylin and eosin (H&E). A “blinded” renal histologist scored the degrees of tubular injury. The magnitude of cell loss and necrotic codes were scored based on five levels. Scores ranged from zero to four based on the percentage of tubules affected (0: <10%; 1: 10 to 25%; 2: 25 to 50%; 3: 50 to 75%; 4: >75%).

Frozen kidney sections at baseline, 24, and 72 h postischemia were stained with a monoclonal antibody specific to neutrophils (Ly-6G, Gr-1, BD Pharmingen). Stained cells in
the corticomedullary zone were counted in a blinded fashion (10 random field were counted at $\times 200$ magnification).

Leukocyte detection using myeloperoxidase assay. Myeloperoxidase assay (MPO) levels were used to detect phagocytes (neutrophil and macrophage) in mouse kidney. Briefly, kidney samples were homogenized (1:20 wt/vol) in ice-cold KPO$_4$ buffer. Samples were spun at 17,000 $g$ for 30 min at 4°C, and pellets were washed and spun an additional two times. Then 0.5% hexadecyltrimethylammonium bromide-10 mM EDTA was added to the remaining pellet (6:1). Suspensions were sonicated and freeze-thawed three times, then $\frac{1}{10}$ samples were incubated at 4°C for 15 min and addition of assay buffer (4:1), supernatants were measured for MPO. Change in absorbance over 3.5 min was recorded at 460 nm. One unit of MPO was defined as a change of absorbance of one per minute.

Results were expressed as percent baseline of units MPO per gram of protein that was detected using a bicinchoninic assay (Pierce Chemical, Rockport, IL).

Renal mRNA cytokine expression. Cellular RNA was isolated from snap-frozen kidneys at 0, 24, and 72 h postischemia using TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA). The quantity of RNA was evaluated by the presence of GAPDH housekeeping RNA and analyzed as a percent increase from “0” hour samples. A ribonuclease protection assay (RPA) was used to characterize the proinflammatory gene expression. We used the mouse-specific multiple cytokine assays RIBOQUANT from Pharmingen. Focus was on the following cytokines: IL-1,$\beta$, TNF-$\alpha$, ICAM-1, and IL-6 based on previous studies implicating them as potential mediators of renal IRI (13).

Statistics. One-way ANOVA was used for the comparison among the group of data, using SigmaStat software (SPSS Science, Chicago, IL). Data are expressed as means ± SE. A $P$ value <0.05% was considered to be significant.

RESULTS

Functional characteristics of $T$ cells from STAT4$^{-/-}$ and STAT6$^{-/-}$ mice. ICCS was performed to phenotype mice that were used in each experimental group. $T$ cells from STAT4$^{-/-}$ mice expressed reduced IFN-$\gamma$ and increased IL-4, whereas those from STAT6$^{-/-}$ mice indeed expressed increased IFN-$\gamma$ and reduced IL-4 (Fig. 1).

Renal function postischemia of STAT4$^{-/-}$ and STAT6$^{-/-}$ mice. STAT4$^{-/-}$ mice had markedly higher serum creatinine compared with Balb/c wild type at 24, 48, and 72 h postischemia ($P < 0.05$). STAT4$^{-/-}$ mice, however, were only modestly protected from renal IRI compared with Balb/c wild type at 24 h postischemia and not at other time points (Fig. 2).
Tubular injury scoring of STAT4- and STAT6-deficient mice. H&E-stained kidney tissues postischemia were scored to determine the severity of tubular injury. STAT6−/− mice had significantly higher tubular injury scores compared with wild-type mice. Tubular injury in STAT4−/− mice was significantly better than STAT6−/− mice but comparable to wild type (Fig. 3).

Polymorphonuclear cell counts. Polymorphonuclear cell counts were evaluated by specific staining to determine whether enhanced infiltration in STAT6−/− mice was a potential mechanism of worse injury postischemia. Although PMN counts increased postischemia, this was similar in both STAT-deficient groups and wild-type mice at both 24 and 72 h postischemia (Fig. 4A).

MPO level. MPO levels in kidney tissue were measured as an alternative approach to assess whether the STAT6 deficiency led to worse injury by augmenting phagocyte (macrophage and neutrophil) infiltration postischemia. MPO levels also increased postischemia but similarly in STAT-deficient and wild-type mice (Fig. 4B).

Proinflammatory gene expression. IL-1β, TNF-α, ICAM-1, and IL-6 have been implicated as mediators of renal IRI (Fig. 5). As previously described (13), gene expression of these molecules measured by RPA increased from baseline postischemia; however, there was no significant difference in the regulation of these genes between the STAT-deficient and wild-type mice.

IL-4- and IFN-γ-deficient mice. In view of the significant reduction in IL-4 production by T cells in the STAT6−/− mice, we hypothesized that IL-4 deficiency was a potential mechanism by which the STAT6−/− mice had the worsened postischemic response. IL-4-
deficient mice also demonstrated a markedly worse functional and histological response after IRI (Figs. 6 and 7). IFN-γ-deficient mice were studied because of the deficiency in IFN-γ in STAT4−/− mice. The IFN-γ-deficient mice had similar degrees of tubular injury and renal dysfunction postischemia as wild-type mice.

**DISCUSSION**

These data demonstrate a major protective role of the STAT6 pathway in renal IRI. Given the deficiency of IL-4 in the STAT6−/− mouse, and similar responses to renal IRI between STAT6−/− mice and IL-4−/− mice, IL-4 deficiency is a likely mechanism to explain the observations in the STAT6 knockout. Renal neutrophil and macrophage infiltration, as well as upregulation of proinflammatory genes such as ICAM-1, are unlikely to underlie the worse renal outcome in the STAT6−/− mice. In contrast, STAT4−/− conferred limited protection from IRI, restricted to modest early functional improvement. IFN-γ deficiency in STAT4−/− was mimicked in the IFN-γ−/− mice, with similar early outcomes to wild type after renal IRI.

A number of groups recently demonstrated a role for T cells in renal IRI (2, 4, 18, 23, 28). Studies in several models of IRI in other organs, such as the liver, intestine, lung, and brain, also support a pathogenic role of T cells (8, 14, 19, 32). However, the mechanisms underlying these effects have not been elucidated (17). One approach in the exploration of the T cell involvement in renal IRI is to examine whether the T cell involvement is via the CD4 cell polarization model into Th1 and Th2 cytokine-producing cells. Proinflammatory cytokines involved in T cell polarization, such as IFN-γ, IL-6, and IL-10, have been reported to be upregulated in postischemic kidneys from wild-type animals (6, 13). When CD4 “helper” T cells functionally differentiate into Th1 and Th2 cells, they require the specific STAT proteins, STAT4 and STAT6. The STAT4 pathway is activated by the conjugation of IL-12 and IL-12R on Th1 cells followed by IFN-γ production. The STAT6 pathway is activated by the conjugation of IL-4 and IL-4R on Th2 cells followed by production of IL-4, IL-5, IL-6, IL-10, and IL-13. IFN-γ promotes Th1 effector functions and inhibits Th2 effector cell functions, whereas IL-4 promotes Th2 functions and inhibits Th1 functions. In a variety of disease models, such as asthma, leprosy, and transplant rejection, T cells are skewed into Th1 or Th2 pathways (1, 27).

On the basis of the hypothesis that the Th1 immune response pattern would be deleterious in renal IRI and Th2 protective, we originally compared the responses to renal IRI in Balb/c mice compared with C57BL6/6. This is based on their relative tendencies to express the Th2 and Th1 phenotypes, respectively (7). Although we found a mild susceptibility to IRI in C57BL/6 mice, these data only suggested the possibility of this paradigm in IRI (3). With the availability of specific knockouts of the key T cell cytokine-polarizing STAT4 and STAT6 genes, this question could now be addressed more directly.

We initially confirmed that the STAT4 mice that we used had a deficiency in CD4 T cell polarization into Th1 cells using ICCS of T cells. The marked impairment in IFN-γ and increased IL-4 demonstrated this. We then evaluated STAT6-deficient CD4+ cells and found the inverse T cell phenotype. Proceeding with renal IRI studies, we found a very modest but reproducible functional protection from renal IRI in the STAT4−/− mice, although histological protection was not seen. We interpreted the mild functional protection in STAT4−/− as likely minor and was not sufficient to be reflected in the visual tubular injury assessment. Alternatively, we cannot exclude a mild “functional” effect on renal blood flow. We had been focusing on identifying deleterious pathways in renal IRI and did

**Fig. 6.** Serum creatinine postischemia in IL-4−/− or IFN-γ−/− deficient mice. IL-4-deficient mice had significantly worse renal function compared with strain-matched wild-type controls at 24 h postischemia. IFN-γ-deficient mice were similar to wild-type postischemia. *P < 0.05 vs. Balb/c control mice (n = 4; ○, Balb/c wild type; △, IFN-γ−/−; □, IL-4−/−).

**Fig. 7.** Tubular injury score for IL-4−/− and IFN-γ−/− deficient mice postischemia. H&E-stained kidney tissues at 72 h postischemia were scored for the level of tubular injury in a blinded fashion. IL-4−/− mice displayed significantly more severe injury compared with wild-type and IFN-γ−/− mice, which were comparable. *P < 0.05.
not expect the unusually severe renal injury phenotype in the STAT6−/− mice, suggesting that this pathway is protective in renal IRI. The markedly increased functional injury in the STAT6−/− mice corresponded to their enhanced tubular injury postischemia, and we therefore turned our focus to this potentially protective pathway.

We then explored potential effector mechanisms by which the STAT6−/− mice had enhanced injury. In view of previous data implicating the neutrophil as being a participant in postschismic renal injury (12), we measured neutrophil influx into postschismic kidney in the wild-type and STAT-deficient mice using specific monoclonal staining. We did not observe a significant difference in the postschismic neutrophil influx between groups. We also used the alternative technique of renal MPO measurement, which primarily detects infiltrating macrophages, but also neutrophils in a more observer-independent fashion than cell counting (31). However, although MPO levels increased in all groups postschismic, they did not correlate with the significantly worse renal function in the STAT6−/− mice. We then measured gene expression of IL-1β, TNF-α, ICAM-1, and IL-6, which have all been implicated as mediators of renal IRI. We used an RPA technique that is established to detect increases in these genes in the current model (13) as well as associate protective changes with an intervention (29). Again, although these genes increased postschismic, their increase did not correlate with the changes in the STAT6−/− mice.

A major characteristic phenotype of the STAT6−/− mouse is the deficiency of T cell production of IL-4. We therefore hypothesized that IL-4 deficiency could be a major mechanism by which the STAT6−/− mice had worse injury after renal IRI. We therefore studied IL-4-deficient mice, which were subsequently found to have a marked worsening of renal function and structure postschismic. The finding that the major “Th2” cytokine IL-4 is likely a protective factor in renal IRI is consistent with the finding by Star et al. (5) that another “Th2” cytokine, IL-10, is also protective in renal IRI. IFN-γ deficiency, however, did not confer a protective phenotype.

Our findings of the enhanced renal injury in the STAT6−/− mouse are consistent with work in the liver using a different approach: IL-4, administered to promote the STAT6 gene, resulted in protection from postschismic liver injury (11, 30). The contribution of the STAT4 gene to liver IRI was also found to be more subtle, with liver IRI in STAT4−/− mice revealing the protection only under the circumstance of endogenous IL-12 blockade (11). The lesser role of STAT4 compared with STAT6 in liver IRI is consistent with our current findings in the kidney. Recently, the STAT6 and STAT4 injury paradigm in liver IRI has been linked to heme oxygenase production (20). It is also important to note that although IL-4 is a prototypic Th2 CD4 cell-secreted cytokine, IL-4 can also be made by NK cells, mast cells, basophils, and undifferentiated T cells (15). In preliminary studies to identify the cell source of IL-4 in ischemia using T cell-adoptive transfer methods, it appeared that the T cell might be a partial source (data not shown).

The identification of the protective role of the STAT6 pathway in renal IRI unveils a novel area of investigation in ARF. In addition, these data suggest the existence of a protective Th2 paradigm in ischemic renal injury. Modification of this signaling pathway and modifying IL-4 responses could lead to new therapeutics for IRI.

DISCLOSURES

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