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Research Article

Th1 and Th2 Related Cytokines Influence Systemic Inflammatory Responses in Renal Injuries Induced by Ischemia and Reperfusion

Vanessa Nunes de Paiva[1*], Rafael Assumpção Larocca[2], Rebecca Monteiro, M. M.[2], Patrícia Semedo[3], Marlene dos Reis, A.[4], Alvaro Pacheco-Silva[3] & Niels Câmara, O. S.[2,3]

[1]*Institute of Reconstructive Neurobiology, University of Bonn, Life & Brain Centre, Bonn, Germany*

[2]*Laboratory of Transplantation Immunobiology, Department of Immunology, University of São Paulo, São Paulo, Brazil*

[3]*Laboratory of Clinical and Experimental Immunology, Nephrology Division, Department of Medicine, Federal University of São Paulo UNIFESP-EPM, São Paulo, Brazil*

[4]*Department of Pathology, Federal university of Uberaba, Minas Gerais, Brazil*

[*]**Correspondence to:** Dr. Vanessa Nunes de Paiva, Institute of Reconstructive Neurobiology, University of Bonn, Life & Brain Centre, Bonn, Germany.

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Abstract

Renal ischemia/reperfusion injury (IRI) is the major cause of acute kidney injury (AKI) in native and transplanted kidneys, with a complex pathogenesis involving leukocyte infiltration, and release of proinflammatory mediators by tubular cells. Recent evidences have shown a critical role of the $CD4^+$ T cells, and the balance between Th1/Th2 cells as possible effector mechanism. In order to evaluate the role of Th1/Th2 immune response in this system, IL-4, IL-10, IL-12 deficient mice and IL-12/IL-10 double deficient mice were used. Moreover, for adoptive transference studies

we generated chimeric mice on C57BL/6 background. Data was collected 24 hours post IRI. Renal function was evaluated by serum urea measurement and renal morphometric histology analyses. Gene expression of IL-6, MCP-1 and HO-1 were also investigated. Strikingly, IL-4, IL-10 and IL-12/IL-10 KO deficient animals presented higher renal dysfunction when compared to controls, followed by higher expression of HO-1, IL-6 and MCP-1. Conversely, IL-12 KO and (IL-12 KO > WT) chimeric mice were absolutely protected from IRI. We also observed in the (IL-12 KO > WT) chimeric mice a reduction in urea levels, a better renal outcome and down-regulation in IL-6 expression when compared with WT > WT chimeras, suggesting an indirect role of Th1 response. Moreover, IL-6 was not detected in the serum of IL-12 deficient mice while neutrophil-recruiting chemokine KC was elevated in IL-4, IL-10 and IL-2/IL-10 deficient mice. Yet, IL-4 was absent in IL-10 and IL-12/IL-10 deficient mice serum. Thus our results confirm that Th1/Th2 related cytokines are critically involved during renal IRI.

Abbreviations

IRI: Ischemia and Reperfusion Injury; ARF: Acute Renal Failure; DGF: Delayed Graft Function; HO-1: Heme Oxygenase-1, BM: Bone Marrow, AKI: acute kidney injury, MCP-1: Monocyte chemotactic protein 1, TLR: Toll Like Receptor, NLR: nucleotide binding oligomerization domain-like receptor.

Introduction

Recent studies have shown an important role of lymphocytes during the evolution and magnitude of the Ischemia and Reperfusion Injury (IRI), although its mechanisms is not well understood [1-10]. Mice deficient for CD4 and CD8 T lymphocytes are protected from IRI, revealing that both CD4 and CD8 T cells participate during the IRI outcome [11,12]. Thymectomized wild-type (WT) mice subsequently depleted of resilient $CD4^+$ T cells presented reduction in renal injury, with almost absence in neutrophil infiltration as confirmed by Pinheiro and colleagues [13].

Evidences using RAG-1 deficient mice shows that lack of B and T cells reduce renal IRI [14,8]. This combined defect is not fully protective, indicating the participation of innate immune cells during the development of the renal IRI [14]. However, it still controversial, once adoptive transfer of wild type $CD4^+$ T cells to RAG-1 deficient mice aggravates the renal damage [7].

CD4 T cells can polarize into distinct subpopulations after priming. This process is dependent of cytokines present in the inflammatory milieu and activation of transcription factors. IL-12 is crucial for Th (T helper) 1 differentiation through Stat4 signaling and activation of the transcription factor T-bet culminating in the production of IFN-γ, that down-regulates IL-4 expression. On the other hand IL-4 induces Th2 differentiation through Stat6 signaling and activation of the transcription factor GATA3, which inhibit IFN-γ. Recently, has been demonstrated that this Th1/Th2 balance plays an important role in the pathogenesis of kidney provoked by IRI [15].

IL-4 deficient mice show significant increase in urea levels on the serum, with irreversible tubular injury post IRI, compared to wild type. Moreover, it is accompanied by higher expression of T-bet, HO-1 and later up-regulation of MCP-1 chemokine in kidneys [15], showing both functional and morphologic renal damage in mice lacking Th2 response. IL-12 deficient mice are not affected, suggesting an important role of Th1 cytokines, such as IL-12 and IFN-γ, in the disease development following IRI [15,16].

Recent studies have determined an important role of Heme oxigenase-1 (HO-1) expression conferring protection to IRI [17,18]. HO-1 is an important immunomodulatory protein with anti-inflammatory and cytoprotective properties [19]. It also known that HO-1 can inhibit CD4 T cell differentiation to Th1 by direct modulation of T-bet expression [20]. We have previously shown that treatment with cobalt protoporphyrin, an HO-1 inducer, reverted renal dysfunction induced by cyclosporine [21]. Herein, we provide evidences showing the importance IL-4 and IL-12 during the development of renal lesion following IRI. We also show that IL-10 play an important role limiting the inflammation, collaborating for a better renal outcome.

Materials and Methods

Animals

C57BL/6 IL-4, IL-10, IL-12 deficient mice, and IL-12/IL-10 double deficient mice were obtained from the University of São Paulo (USP) Animal Facility, São Paulo, Brazil. All procedures were in accordance with the internal Ethical Committee of the Institution.

Experimental Model of Renal IRI

Surgery was performed as previously described [22,23,13]. Mice were anesthetized with a ketamine-xylazine combination. A midline incision was made and both renal pedicles were cross-clamped. Animals were hydrated with saline and kept at 37°C. Forty-five minutes later, the microsurgery clamps were removed. The abdomen was closed in two layers and the animals were placed in single cages, warmed by indirect light. In the Sham group, animals were submitted to surgery except for the clamping procedure.

Body Weight Analysis

Mice were weighed before and 24 hours after renal injury. Weight loss was calculated by difference in total body weight (grams) before and 24 hours after IRI.

Analysis of Renal Function

Serum urea levels were measured for the evaluation of renal functions after IRI. Blood samples were collected 24 hours post-reperfusion from the ocular plexus immediately before euthanasia. Serum urea was measured using the urease enzymatic method (Labtest, São Paulo, Brazil) following manufacture's instructions. Samples were analyzed using a Spectra Max 190 spectrophotometry and the SoftMax® Pro Software (Molecular Devices, New Jersey, USA).

Generation of Chimeric Mice

To generate the Bone Marrow Chimeras, isogenic WT, IL-12 $^{-/-}$, IL-4 $^{-/-}$ mice all from C57BL/6 background were used as previously described [7]. Briefly bone marrow from donor mice was harvested from femur and tibia under sterile conditions. The bone marrow was passed sequentially through a 22-gauge needle followed by three passages through a 25-gauge needle to obtain single cell suspensions. Cells were washed and ressuspended and viable cells were counted. Recipient mice (4-8 week of age; 22-25 g) were irradiated with a non-lethal dose (1.200 rad/20 minutes). Immediately following irradiation, 20 x 10^6 BM cells were injected via IP. The resulting chimeric mice were housed for 6 weeks before experimentation.

Histological Morphometric Analysis

Paraffin embedded sections from kidney samples were stained with hematoxylin and eosin. Renal histomorphometry analyses ware performed by a "blinded" renal histologist that scored the percentage degrees of the tubular necrosis, using optic light microscopy.

Gene Expression Profiles

Total RNA was isolated from kidney tissue using phenol as manufacturer's instructions. RNA concentration was determined by spectrophotometer using Nanodrop® (Nanodrop, Delaware, USA). First strand cDNAs were synthesized using the MML-V reverse transcriptase (Promega, USA). RT-PCR was performed using the SYBR Green real-time PCR assay (Applied Biosystem, USA). Sequences of mouse primers used were: HO-1 (F) 5' TCA GTC CCA AAC GTC GCG GT 3'and (R) 5' GCT GTG CAG GTG TTG AGC C 3'; IL-6 (F) 5´AGG ATA CCA CTC CCA ACA GAC CT 3´and (R) 5´TTT CTC ATT TCC ACG ATT TCC C 3'; MCP-1 (F) 5´TAG AGA TTG AGC TGT CTG CTC ATT C 3´and (R) 5´CCT AAA GTA TGG GCT GGA CTG TTT 3´. Target genes were normalized by the endogenous control (HPRT) (F) 5' CTC AT GAC TGA TTA TGG ACA GGA C 3' and (R) 5' GCA GGT CAG CAA AGA ACT TAT AGC C 3'.

Cytokine and Chemokine Measurements by Bioplex

A Bio-Plex mouse Th1/Th2-Plex cytokine assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to test samples for the presence of 6 cytokines: anti-inflammatory cytokines such as IL-4 and IL-10, pro-inflammatory cytokines such as IL-6 and IL-1β, RANTES and KC. The assay was read on the Bio-Plex suspension array system, and the data were analyzed using Bio-Plex Manager software version 4.0. Standard curves ranged from 32,000 to 1.95pg/mL. Lower values were: IL-4 1.93pg/mL; IL-10 1.92pg/mL; IL-1β 1.94pg/mL; IL-6 1.95pg/mL; RANTES 1.95pg/mL; and KC 2.01pg/mL.

Statistical Analysis

Serum urea data was described as mean ± SD unless otherwise stated. Morphometric and qRT-PCR data were presented as a median and ranges. Parametric and nonparametric tests were performed according to the sample distribution considered normal or not.

Differences among the groups were compared using ANOVA (Kruskal-Wallis test) followed by Dunn's multiple comparison tests. The null hypothesis was rejected when $p<0.05$. All statistical analyses were performed using GraphPad Prism 6.

Results

IL-10 is Important for Limiting Inflammatory Tissue Injury after Renal IRI

The participation of $CD4^+$ T cells causing tissue damage after renal IRI is poorly understood. Data from our group shows that following IRI, IL-4 deficient mice develop a more robust inflammatory response, characterized by infiltration of Th1 cells, with elevated levels of IL-12 and IFN-γ cytokines, culminating for an worse IRI outcome [16]. Here we show that 24 hours post IRI, IL-$12^{-/-}$ mice present better renal outcome, represented by Figure 1) maintenance of weight and; 1 b) lower levels of urea in serum (IL-12: 53.2 ± 0.08 mg/dL, p=0.001), when compared to WT mice (Figure 1a). On the other hand, IL-$4^{-/-}$, IL-$10^{-/-}$ and IL-$10^{-/-}$/IL-$12^{-/-}$ double deficient mice show elevated serum urea levels (IL-4: 201.2 ± 0.03, p=0.15, IL-10: 197.0 ± 0.04, p=0.90 and IL-10/IL-12: 200.3 ± 1.23, mg/dL, p=0.74) when compared with WT mice (198.9 ± 4.8mg/dL, p=0.38) (Figure 1a). We also observed a more pronounced weight loss when compared with WT mice (Figure 1b).

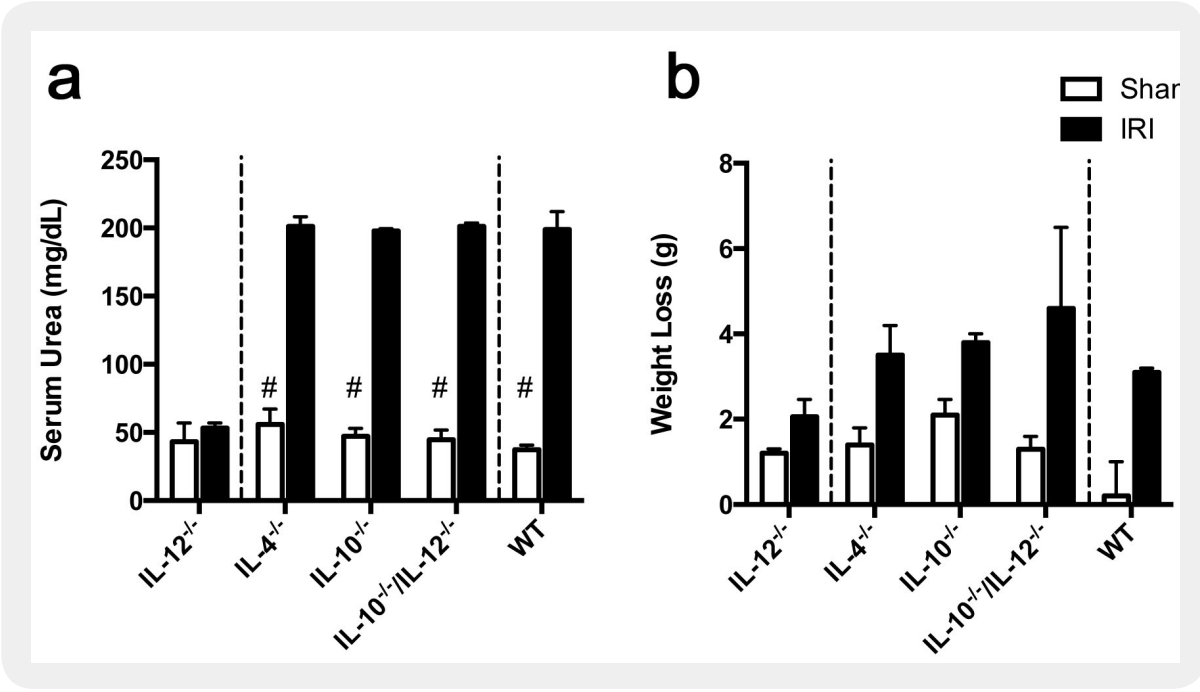

Figure 1: *Weight loss animal and renal function after 24 hour of ischemia*

IL-12 -/- had the lowest weight loss at 24 hour after ischemia (B). Renal function was estimated by serum urea (mg/dL) measured by colorimetric method. IL-12 -/- protector was observed after 24-hour ischemia, while IL-4 -/-, IL-10 -/- and IL-10/IL-12 double -/- was not protector after 24 hour of ischemia. Data of sham operated animals are also demonstrated, wild-type, IL-12 -/-, IL-4 -/-, IL-10 -/- and IL-10/IL-12 double -/- presented values similar to normal, all compared with wild-type animals (A).

In all experiments, serum urea (mg/dL) was measured from samples obtained from ocular plexus animals at 24 hour after ischemia (45 minutes of bilateral clamping), Statistical analyses were performed using ANOVA. # P<0.05.

IL-12 Protects from Acute Tubular Necrosis after IRI

Histological analysis demonstrated that IL-12$^{-/-}$ mice, show reduced tubular necrosis after IRI, compared to WT, 24 hours post ischemia (IL-12: 0%, 0-34,6). The IL-4$^{-/-}$, IL-10$^{-/-}$, IL-10$^{-/-}$/IL-12$^{-/-}$ deficient mice presented more areas with tubular necrosis, when compared to control WT (Figure 2A and B). Moreover, the histologic assessment also revealed that in the of knockout mice, the tubular acute damage reached the medullar area, as compared with WT control group (Figure 2B).

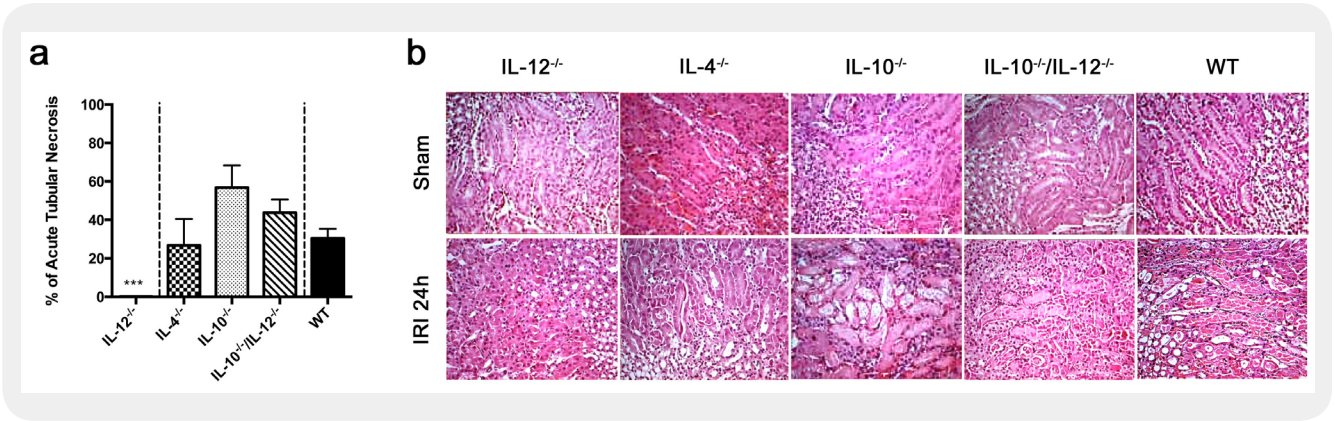

Figure 2: *Mouse kidney tissue after 24 hours ischemia*

Kidney histology in tissue sections stained with H&E was observed at original magnification X 835. Kidney tissue from IL-12 -/- mice after 24 hours ischemia, (B, lower painel), shows lower percentage acute tubular necrosis compared with controls animal wild-type histology 24 hours after ischemia and IL-4$^{-/-}$, IL-10 $^{-/-}$, IL-10/IL-12 double $^{-/-}$ and wild-type after 24 hours ischemia (a and b). IL-12 -/- deficient animals presented a lower percentage of acute tubular necrosis and not presentation tubular regeneration at 24 hours postichemia IRI. Median values of acute tubular necrosis in wild type, IL-12 -/-, IL-4 -/-, IL-10 -/- and IL-10/IL-12 double -/- after 24 hours ischemia. Sham operated animals had no quantifiable acute necrosis and tubular regeneration after ward (not show in the figure), because the percentage is zero, when percentage of ATN these animals was compared wild type. There are statistical differences between IL-12 -/- and Wild-type. Statistical analyses were performed using ANOVA. ***P<0.001.

Deficiency of IL-10 is Associated with Higher Expression of IL-6, MCP-1 and HO-1 in the Kidney

In order to demonstrate the presence of pro-inflammatory cells infiltrating the ischemic kidneys, we performed a qRT-PCR for IL-6, MCP-1 and HO-1. The IL-12$^{-/-}$ mice presented the lower levels of IL-6 expression 24 hours post ischemia, compared to WT control mice (IL-12: 8.15, 5.07-11.55, p=0.005, and WT: 30.13, 22.58-42.40). Conversely, IL-4$^{-/-}$, IL-10$^{-/-}$, IL-10$^{-/-}$/IL-12$^{-/-}$ animals that were not protected against IRI presented a major up-regulation of IL-6 mRNA expression (IL-4: 50, 28.7-104; IL-10: 58.9, 43.2-69; IL-10/IL-12: 20, 13-50; p=0.54) when compared to control animals (Figure 3 a and c). Previously we have observed that IL-12 deletion decreased HO-1 and IL-6 expression, IL-12-/- deficient mice achieved similar results [16].

On the other hand, as the expression of HO-1 has been strongly related to cytoprotection, and its levels positively correlated with tissue stress and injury, we tested for HO-1 mRNA expression in IL-12-/- deficient mice subjected to IRI. We observed lower levels of HO-1 mRNA expression in IL-12$^{-/-}$ deficient animals without statistical difference (1.23, 1.20-1.26, p=0.06), at 24 hours post ischemia. On the other hand, IL-4$^{-/-}$, IL-10$^{-/-}$, IL-10/IL-12$^{-/-}$ double deficient animals, presented a slightly increase in the expression of HO-1 (IL-4: 3.58, 3.75, 3.85; IL-10: 6.78, 6.51, 5.87; and IL10/IL-12: 3.25, 3.68, 3.95, p=0.13) when submitted to IRI, all compared to WT (4.13, 2.39-7.94) (Figure 3 c).

The renal tubular epithelium is able to generate MCP-1, thereby determined by the renal injury induced by leukocyte infiltration I/R kidney. Thus we investigated the levels of MCP-1 post IRI, and we observed that IL-12-/- deficient animals presented significantly lower levels of MCP-1 mRNA expression, 24 hours post ischemia, when compared with IL-10/IL-12-/- deficient animals (p=0,003) (Figure 3 b).

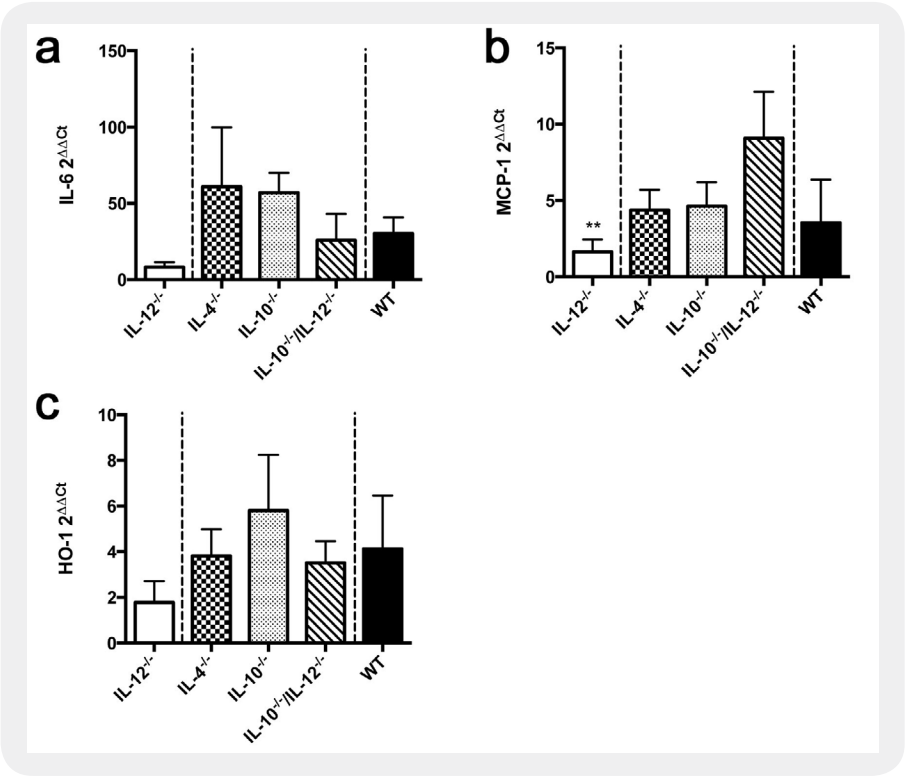

Figure 3: *Pro-inflammatory cytokines, chemokines and cytoprotector gene expression in IL-12 -/-, IL-4 -/-, IL-10 -/-, IL-10/IL-12 double -/- and wild-type animals after 24 hours ischemia*

All molecule expression was measured by real time PCR at 24 hours after ischemia. IL-12 -/- group had lower pro-inflammatory cytokines and chemokines expression (IL-6 and MCP-1), (A and B) and lower citoprotector gene expression (HO-1), (C). Sham operated animals showed neglected values not presented on the graphic all compared wild type. Statistical analyses were performed using ANOVA. *** p<0.0001.

Pro and Anti-Inflammatory Molecules Presence in Serum Th1 and Th2 Deficient Mice after Renal IRI

Pro and anti-inflammatory molecules were measured at 24 hours of reperfusion in IL-12 -/-, IL-4 -/-, IL-10 -/- and IL-10/IL-12 -/- double deficient mice. We observed that the pro-inflammatory cytokines and chemokines and the IL-6, IL-1β, RANTES and KC were significantly reduced by IL-12 -/- deficient mice (Figure 4 a, c, e and f). On the other hand, the anti-inflammatory components IL-4 were not presented with statistical differences in IL-4 -/-, IL-10 -/- and IL-10/IL-12 -/- deficient mice, specific one response Th2 (Figure 4 b) and IL-10 were not presented in IL-10 -/- deficient mice with statistical differences and presented lower levels in IL-4 -/- and IL-10/IL-12 -/- double deficient mice after 24 hours ischemia (Figure 4 d). The pro-inflammatory cytokine IL-1ß and RANTES were increased after 24 hours of renal reperfusion in serum of the Th2 polarization deficient mice (IL-4 and IL-10 deficient and IL-10/IL-12 double deficient). The IL-12 -/- deficient mice are protected from injury.

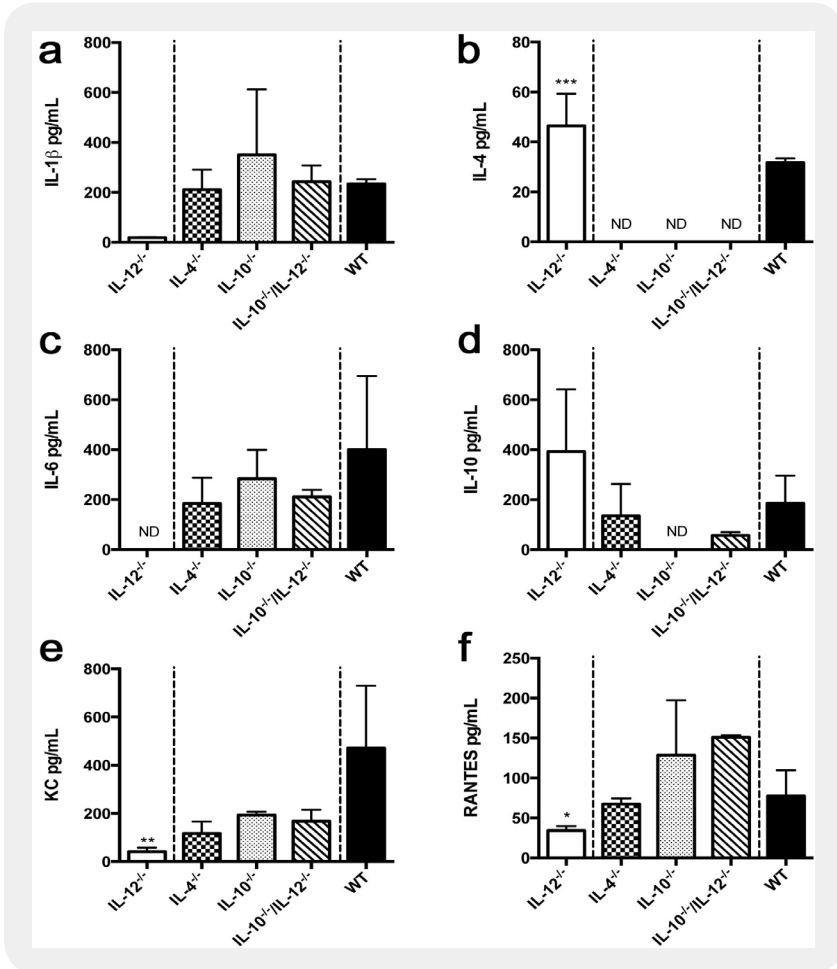

Figure 4: *Pro and anti-inflammatory molecules presence in IL-12 -/-, IL-4 -/-, IL-10 -/-, IL-10/IL-12 double -/- and wild-type animals after 24 hours ischemia*

All molecules are present in serum these animals and were measured by bioplex at 24 hours after ischemia. IL-12 -/- group had lower pro-inflammatory cytokines and chemokines (IL-6, IL-1β, KC and RANTES), (A, C, E and F) had highest anti-inflammatory cytokines (IL-4 and IL-10), (B and D). All compared with wild-type animals. Sham operated animals showed neglected values not presented on the graphic and presented values similar to normal animals. Statistical analyses were performed using ANOVA. * and **P<0.01.

These results suggest that Th2 response contributes significantly to renal IRI; in its absence kidneys are not protected from IRI. These results indirectly implicate cytokines participation one Th1 and Th2 response mediating renal IRI.

The IL-12 Cytokine Confer Protection after Bone Marrow Reconstitution in Wild-Type Mice, and Down Regulate Effect Pro-Inflammatory Cytokine IL-6 after Renal IRI

We sought to determine the specific role of IL-12 and IL-4 cytokines in IRI. Using a total bone marrow these animals we were prepared for adoptive transfer into wild-type mice. We injected bone marrow cells the IL-12-/- and IL-4 deficient mice into wild-type mice via intraperitoneal, the spleen and blood were analyzed for CD3 +, CD4 + and CD8+ T lymphocyte after the bone marrow were replaced by FACS analyses. The resulting chimeric mice were housed for 6 weeks before experimentation. Reconstitution percentage of $CD3^+$, $CD4^+$ and $CD8^+$ T cells was 82%, 84% and 91% respectively. Prior to cell transference those numbers were 0,01%, 0,02% and 0,05%, respectively (data not show).

The IL-12 -/- → WT chimeric mice presented better renal function and lower weight loss after renal ischemia and reperfusion injury compared to control wild type mice (WT → WT). IL-12 -/- → WT chimeric mice showed decrease in serum urea at 24 hours post ischemia (IL-12 -/-: 0.60 ± 0.04 mg/dL, p=0.04), when compared to wild type (WT → WT). (Figure 5 b and c). Knockout sham-operated animals presented similar serum urea levels to the normal animals, with statistical differences. When we performed the opposite experiment where the bone marrow of WT mice were transferred to IL-12 -/- mice showed lower serum urea levels and lower weight loss compared with wild-type mice at 24 hours post IRI (Figure 5 b and c).

On the other hand, IL4 -/- → WT and WT → IL-4 -/- chimerics mice showed higher serum urea levels and higher weight loss compared with wild-type mice at 24 hours post ischemia (Data not show).

These results indicate that kidneys from IL-12 -/- deficient mice were protected from IRI and that adoptive transfer provides additional tissue protection, suggesting that the protective effect of IL-12 could be mediated through one defect Th1 response.

Effect Pro-Inflammatory Cytokine IL-6 in Chimera Mice After Renal IRI

The IL-12 -/- → WT chimerics mice presented lower levels of IL-6 expression at 24 hours postischemia, compared to wild-type (WT → WT), (IL-12 KO: 8.15, 5.07-11.55, p=0.027; IFN-g/IL-12 KO: 19.69, 7.60-29.51, p=0.30; wild-type: 30.13, 22.58-42.40). Conversely, WT → IL-12 -/-, IL-4 -/- → WT and WT → IL-4 -/- that were not protected against IRI presented a major up-regulation of IL-6 mRNA expression still when compared to control animals (WT → WT) (Figure 5 a).

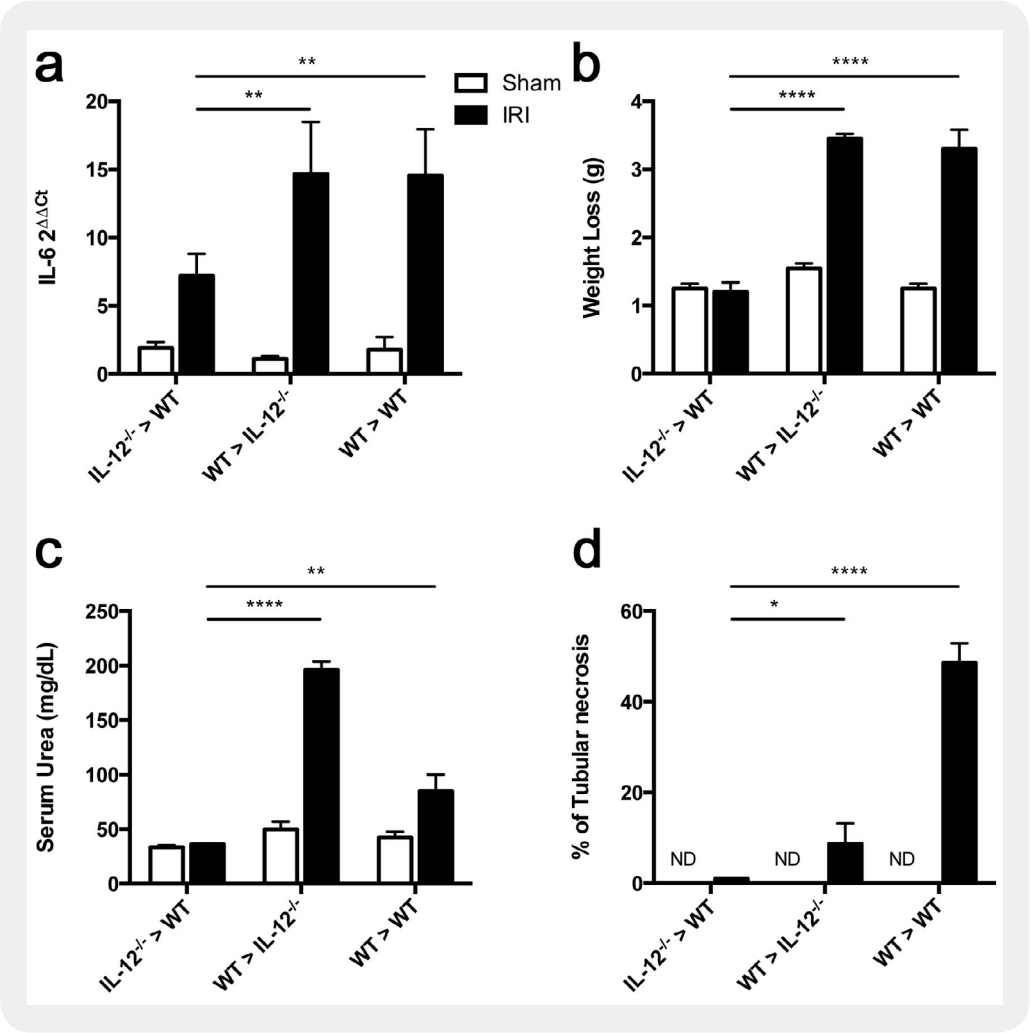

Figure 5: *Effect of renal IRI on weight loss and serum urea in chimeric mice*

IL-12 -/- → WT had lowest weight loss, but WT → IL-12 -/- had highest weight loss when compare WT → WT controls chimeric mice (B). The IL-4 -/- → WT and WT → IL-4 -/- chimeric mice had highest weight loss at 24 hours after ischemia (C and D). Renal function was estimated by serum urea (mg/dL) measured by colorimetric method. IL-12 -/- → WT protector function and WT → IL-12 -/-, IL-4 -/- → WT and WT → IL-4 -/- (F, G and H) are not protector renal function when compare WT → WT control chimeric mice was observed after 24 hour ischemia. Data of sham operated animals are also demonstrated, IL-12 -/- → WT, WT → IL-12 -/-, IL-4 -/- → WT and WT → IL-4 -/- presented values similar to normal, all compared with wild-type animals. In all experiments, serum urea (mg/dL) was measured from samples obtained from ocular plexus animals at 24 hour after ischemia (45 minutes of bilateral clamping), Statistical analyses were performed using ANOVA. * $P<0.05$.

Discussion

Renal IRI, is accompanied of up-regulation of proinflammatory molecules on kidneys, that drives the immune response to a Th1 pattern, such as IFN- γ and IL-12 [24]. We observed in IL-12 deficient animals following IRI, a reduced inflammation with reduced acute tubular necrosis. On the other hand, when we

observed animals depleted of IL-4, IL-10 or IL-10/IL-12 cytokines, protection was lost, similar to control WT. Marques and colleagues have demonstrated the involvement of Th1 and Th2 cytokines in renal IRI, and that a Th2 immune response was not related to protective tissue injury [15]. In addition, Yokota and colleagues using animals deficient to STAT-4, lacking the ability to CD4 T cells respond to IL-12 stimulus, presented protective response from renal IRI [25].

Herein, protection observed in IL-12 deficient animals, clearly demonstrates that the abrogation of a Th1 response is deleterious to the renal IRI. We also showed that the protection observed in IL-12$^{-/-}$ mice was lost when we used the IL-12$^{-/-}$/IL-10$^{-/-}$ mice, suggesting an import role of IL-10 regulating the immune response even on the absence of IL-12.

It is known that IL-12$^{-/-}$ mice have elevated IL-10 plasma levels, culminating in an inability to adequately remove bacteria after infection. In contrast, IL-10 deficient mice when infected with *Pneumocystis carinii,* have high concentrations of IFN-γ and IL-12, demonstrating an intimate association between these cytokines [26].

In all groups, data from kidney morphometric analysis were in agreement with the biochemical and qRT-PCR data. In summary, our data demonstrate that the presence of IL-12, which could be associated with a Th1 immune response, is harmful to the kidney following IRI. In accordance with this, the level of both IL-6 and MCP-1 mRNA levels were decreased in the IL-12$^{-/-}$ group. Moreover, the animals lacking IL-4, IL-10 and IL-10/IL-12 cytokines showed higher expression levels of IL-6. Thus, our data show that IL-6 is involved in the process of renal IRI. When we evaluate the levels of MCP-1, which is an important chemokine for macrophage infiltration, we observed that animals deficient in both IL-10/IL-12 lost protection. These findings are consistent with the morphometric analysis, where we observed a higher degree of renal dysfunction and macrophage infiltration (data not show). This data enforces the role of IL-10 in control the inflammatory response and protection to renal IRI.

Furthermore, we observed a slightly down regulation of HO-1 in the IL-10/IL-12 deficient mice. It is in accordance with the literature, where some studies have shown a negative feedback between the expression of MCP-1 and HO-1 [19]. The early expression of higher levels of HO-1 is accompanied by inhibition of MCP-1 expression. Thus, these data reinforce the protective influence of IL-10, as Lee and colleagues demonstrated that IL-10 acts on macrophages to produce HO-1 [27]. It would explain our findings, where in the absence of IL-10 and IL-12 we observed higher expression of MCP-1.

The induction of HO-1 expression post-acute renal failure (ARF) has been described in experimental models of induced by I/R [19]. It is known that the inhibition of HO-1 expression in the kidneys subjected to ischemic insult, results in a worsening of renal function. Thus, HO-1 expression seems to have a crucial effect in the cytoprotection of renal tissue subjected to I/R [20,21].

Therefore, the findings observed in mice deficient in IL-4 and IL-10 (representing a defect in the Th2 immune response pathway), were directly related to a higher degree of morphologic damage and kidney dysfunction, compared with mice deficient in IL-12 (representing a defect of the Th1 pathway). Strengthening our data, we performed the adoptive transfer of total bone marrow cells from IL-12 deficient mice to previously non-lethal irradiated wild-type mice. What we observed was a complete protection of renal injury post I/R, favouring the hypothesis of that IL-12 drives the response through a Th1 pathway, resulting in IRI. This protection was confirmed by histological data and the low expression of IL-6.

Few studies show results with chimeric animals underwent injury of I/R. Okusa and colleagues using Rag-1 deficient mice, observed that these animals were protected from injury post kidney I/R. This effect was abolished when animals were reconstituted with CD4+ T lymphocytes [7]. Another group published that knockout animals for the CCR2 chemokine were protected from injury from I/R kidney, but after the adoptive transfer of total bone marrow cells from wild animals, the lesion was restored.

Finally, we examine the presence of cytokines pro-and anti-inflammatory serum of normal animals ischemia. The cytokine IL-4, mainly involved with the Th2 profile, IL-6, a pro-inflammatory cytokines, KC, a chemokine that causes chemotaxis to neutrophils and are produced mainly by macrophages, RANTES are able to activate T cells, triggering the synthesis of IL-2 and IFN-γ without the presence of antigens, and this activation was mediated by protein-kinases and that this property was not shared by other chemokines, other pro-inflammatory cytokine IL-1β and anti-inflammatory cytokines such as IL-4 and IL-10. Detected higher concentration of cytokine IL-6 in serum of animals that were not protected from injury of I/R, as the deficient animals to IL-4, IL-10 and double deficient IL-10/IL-12, whereas animals had not protected the presence of IL-6 in serum. These data corroborate the data found in the analysis of mRNA expression of IL-6.

The chemokine KC was shown the highest concentration in the serum of animals that were not protected of injury from I/R kidney, indicating that these animals may have a greater infiltration of neutrophils in the kidney in 24 hours. Moreover, the concentration the cytokine IL-4 and IL-10 had higher in serum of animals protected from injury from I/R kidney, as in the deficient animals to IL-12 and absent in deficient animals to IL-4, IL-10 and double deficient for IL-10/IL-12. These results only reinforce the idea that the IRI the pro and anti-inflammatory cytokines have systemic function.

Taken together, our data show that IL-12 is the major cytokine involved during the process of renal failure following kidney I/R and IL-10 seems to be involved in the modulation of the Th1 immune response. Our results encourage new studies on the activation of macrophages and its involvement with $CD4^+$ T lymphocytes during the IRI outcome.

Conclusions

Such findings favour the hypothesis of the deleterious effects of Th1 cells with profile and the protective role of Th2 cells in ischemic renal injury. These novel insights may provide basis for the better pathophysiological understanding, as well as for development of preventive and therapeutic methods for the ischemic acute renal failure.

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Authors' Disclosure

The authors declare no conflicts of interest.

Bibliography

1. Bajwa, A., Huang, L., Ye, H., Dondeti, K., Song, S., *et al.* (2012). Dendritic cell sphingosine 1-phosphate receptor-3 regulates Th1-Th2 polarity in kidney ischemia-reperfusion injury. *J Immunol., 189*(5), 2584-2596.

2. Kinsey, G. R., Huang, L., Vergis, A. L., Li, L. & Okusa, M. D. (2010). Regulatory T cells contribute to the protective effect of ischemic preconditioning in the kidney. *Kidney Int., 77*(9), 771-780.

3. Ko, G. J., Linfert, D., Jang, H. R., Higbee, E., Watkins, T., *et al.* (2012). Transcriptional analysis of infiltrating T cells in kidney ischemia-reperfusion injury reveals a pathophysiological role for CCR5. *Am J Physiol Renal Physiol., 302*(6), F762-773.

4. Lai, L. W., Yong, K. C. & Lien, Y. H. (2012). Pharmacologic recruitment of regulatory T cells as a therapy for ischemic acute kidney injury. *Kidney Int., 81*(10), 983-992.

5. Loverre, A., Divella, C., Castellano, G., Tataranni, T., Zaza, G., *et al.* (2011). T helper 1, 2 and 17 cell subsets in renal transplant patients with delayed graft function. *Transpl Int., 24*(3), 233-242.

6. Tadagavadi, R. K., Wang, W. & Ramesh, G. (2010). Netrin-1 regulates Th1/Th2/Th17 cytokine production and inflammation through UNC5B receptor and protects kidney against ischemia-reperfusion injury. *J Immunol., 185*(6), 3750-3758.

7. Day, Y. J., Huang, L., Ye, H., Li, L., Linden, J. & Okusa, M. D. (2006). Renal ischemia-reperfusion injury and adenosine 2A receptor-mediated tissue protection: the role of $CD4^+$ T cells and IFN-gamma. *J Immunol., 176*(5), 3108-3114.

8. Huang, Y., Rabb, H. & Womer, K. L. (2007). Ischemia-reperfusion and immediate T cell responses. *Cell Immunol., 248*(1), 4-11.

9. Ascon, D. B., Ascon, M., Satpute, S., Lopez-Briones, S., Racusen, L., Colvin, R. B., Soloski, M. J. & Rabb, H. (2008). Normal mouse kidneys contain activated and CD3+CD4- CD8- double-negative T lymphocytes with a distinct TCR repertoire. *J Leukoc Biol., 84*(6), 1400-1409.

10. Linfert, D., Chowdhry, T. & Rabb, H. (2009). Lymphocytes and ischemia-reperfusion injury. *Transplant Rev (Orlando), 23*(1), 1-10.

11. Rabb, H., Daniels, F., O'donnell, M., Haq, M., Saba, S. R., Keane, W. & Tang, W. W. (2000). Pathophysiological role of T lymphocytes in renal ischemia-reperfusion injury in mice. *Am J Physiol Renal Physiol., 279*(3), F525-531.

12. Yokota, N., Daniels, F., Crosson, J. & Rabb, H. (2002). Protective effect of T cell depletion in murine renal ischemia-reperfusion injury. *Transplantation, 74*(6), 759-763.

13. Pinheiro, H. S., Camara, N. O., Noronha, I. L., Maugeri, I. L., Franco, M. F., Medina, J. O. & Pacheco-Silva, A. (2007). Contribution of CD4+ T cells to the early mechanisms of ischemia- reperfusion injury in a mouse model of acute renal failure. *Braz J Med Biol Res., 40*(4), 557-568.

14. Burne-Taney, M. J., Yokota-Ikeda, N. & Rabb, H. (2005). Effects of combined T- and B-cell deficiency on murine ischemia reperfusion injury. *Am J Transplant., 5*(6), 1186-1193.

15. Marques, V. P., Goncalves, G. M., Feitoza, C. Q., Cenedeze, M. A., Fernandes Bertocchi, A. P., *et al.* (2006). Influence of TH1/TH2 switched immune response on renal ischemia-reperfusion injury. *Nephron Exp Nephrol., 104*(1), e48-56.

16. De Paiva, V. N., Monteiro, R. M., Marques Vde, P., Cenedeze, M. A., Teixeira Vde, P., Dos Reis, M. A., Pacheco-Silva, A. & Camara, N. O. (2009). Critical involvement of Th1-related cytokines in renal injuries induced by ischemia and reperfusion. *Int Immunopharmacol., 9*(6), 668-672.

17. Ferenbach, D. A., Ramdas, V., Spencer, N., Marson, L., Anegon, I., Hughes, J. & Kluth, D. C. (2010). Macrophages expressing heme oxygenase-1 improve renal function in ischemia/reperfusion injury. *Mol Ther., 18*(9), 1706-1713.

18. Lu, J. X., Yan, C. Y., Pu, J. X., Hou, J. Q., Yuan, H. X. & Ping, J. G. (2010). [Gene transfer-induced human heme oxygenase-1 over-expression protects kidney from ischemia-reperfusion injury in rats]. *Zhonghua Yi Xue Za Zhi., 90*(46), 3300-3304.

19. Camara, N. O. & Soares, M. P. (2005). Heme oxygenase-1 (HO-1), a protective gene that prevents chronic graft dysfunction. *Free Radic Biol Med., 38*(4), 426-435.

20. Feitoza, C. Q., Goncalves, G. M., Bertocchi, A. P., Wang, P. W., Damiao, M. J., *et al.* (2007). A role for HO-1 in renal function impairment in animals subjected to ischemic and reperfusion injury and treated with immunosuppressive drugs. *Transplant Proc., 39*(2), 424-426.

21. Goncalves, G. M., Cenedeze, M. A., Feitoza, C. Q., De Paula, C. B., Macusso, G. D., Pinheiro, H. S., Teixeira Vde, P., Dos Reis, M. A., Pacheco-Silva, A. & Camara, N. O. (2006). Heme oxygenase 1 and renal ischemia and reperfusion injury: the impact of immunosuppressive drug. *Int Immunopharmacol., 6*(13-14), 1966-1972.

22. Kelly, K. M. (1996). Does increasing oxygen delivery improve outcome? Yes. *Crit Care Clin., 12,* 635-644.

23. Rabb, H. (1996). Role of leukocytes and leukocyte adhesion molecules in renal ischemic-reperfusion injury. *Front Biosci., 1,* e9-14.

24. Tilney, N. L., Paz, D., Ames, J., Gasser, M., Laskowski, I. & Hancock, W. W. (2001). Ischemia-reperfusion injury. *Transplant Proc., 33,* 843-844.

25. Yokota, N., Burne-Taney, M., Racusen, L. & Rabb, H. (2003). Contrasting roles for STAT4 and STAT6 signal transduction pathways in murine renal ischemia-reperfusion injury. *Am J Physiol Renal Physiol., 285*(2), F319-325.

26. Qureshi, M. H., Harmsen, A. G. & Garvy, B. A. (2003). IL-10 modulates host responses and lung damage induced by Pneumocystis carinii infection. *J Immunol., 170*(2), 1002-1009.

27. Lee, T. S. & Chau, L. Y. (2002). Heme oxygenase-1 mediates the anti-inflammatory effect of interleukin-10 in mice. *Nat Med., 8,* 240-246.