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Dendritic Cell Sphingosine 1-Phosphate Receptor-3 Regulates Th1–Th2 Polarity in Kidney Ischemia–Reperfusion Injury

Amandeep Bajwa,*† Liping Huang,*‡ Hong Ye,*† Krishna Dondeti,*† Steven Song,*† Diane L. Rosin,*†‡ Kevin R. Lynch,*‡ Peter I. Lobo,*† Li Li,*† and Mark D. Okusa*†

Dendritic cells (DCs) are central to innate and adaptive immunity of early kidney ischemia–reperfusion injury (IRI), and strategies to alter DC function may provide new therapeutic opportunities. Sphingosine 1-phosphate (SIP) modulates immunity through binding to its receptors (SIP1–5), and protection from kidney IRI occurs in SIP3-deficient mice. Through a series of experiments we determined that this protective effect was owing in part to differences between SIP3-sufficient and -deficient DCs. Mice lacking SIP3 on bone marrow cells were protected from IRI, and SIP3-deficient DCs displayed an immature phenotype. Wild-type (WT) but not SIP3-deficient DCs injected into mice depleted of DCs prior to kidney IR reconstituted injury. Adoptive transfer (i.e., i.v. injection) of glycolipid (Ag)-loaded WT but not SIP3-deficient DCs into WT mice exacerbated IRI, suggesting that WT but not SIP3-deficient DCs activated NKT cells. Whereas WT DC transfers activated the Th1/IFN-γ pathway, SIP3-deficient DCs activated the Th2/IL-4 pathway, and an IL-4–blocking Ab reversed protection from IRI, supporting the concept that IL-4 mediates the protective effect of SIP3-deficient DCs. Administration of SIP3-deficient DCs 7 d prior to or 3 h after IRI protected mice from IRI and suggests their potential use in cell-based therapy. We conclude that absence of DC SIP3 prevents DC maturation and promotes a Th2/IL-4 response. These findings highlight the importance of DC SIP3 in modulating NKT cell function and IRI and support development of selective SIP3 antagonists for tolerizing DCs for cell-based therapy or for systemic administration for the prevention and treatment of IRI and autoimmune diseases. The Journal of Immunology, 2012, 189: 2584–2596.

The pathogenesis of kidney injury following kidney ischemia–reperfusion (IR) involves a complex interaction between altered microcirculatory hemodynamics, endothelial and epithelial cells, and infiltration of immune cells (1, 2). Dendritic cells (DCs), the major subset of leukocytes in the kidney (3–5), contribute to innate and adaptive immunity of kidney IRI (6) through activation of NKT cells and T cells (7–9). NKT cells, a specialized innate T cell subpopulation, recognize glycolipid Ags presented by CD1d, a nonclassical MHC class I (MHCIID) molecule (10). Additionally, IL-12 and IL-23 release from DCs (11, 12), interaction of CD40/CD40L, and CD1d/MHCII molecule (10). Additionally, IL-12 and IL-23 release from DCs (11, 12), interaction of CD40/CD40L, and CD1d/glycolipid presentation lead to NKT cell activation (11, 12) in the early innate immune response of IRI (3, 7). Infiltration and activation of IFN-γ–producing NKT cells leads to the downstream production of CXCL1, CXCL1-mediated neutrophil infiltration (7), and neutrophil-dependent IFN-γ and IL-17 production (12). IL-18 mediates a pro-Th2–like response by enhancing NKT cell IL-4 production independently of IL-12 (13), indicating that different conditions skew NKT cell Th1–Th2 polarity. NKT cell activation has beneficial or detrimental effects depending on activation-induced polarization of Th1 or Th2 responses. Unlike IRI, activation of NKT cells in autoimmune diseases results in amelioration of disease by shifting the balance toward a Th2 response. Sphingosine 1-phosphate (SIP1), a sphingolipid that is produced by phosphorylation of sphingosine by sphingosine kinases, is the natural ligand for a family of five G protein-coupled receptors (SIP1–5) and evokes diverse cellular signaling responses (14–16). Most of the SIP effects are mediated through the SIP receptor family, which includes the ubiquitously expressed SIP1, SIP2, and SIP3 subtypes (17). T cells express SIP1 at a higher amount compared to SIP4 (18, 19), and human DCs express SIP1–4 (20).

Tissue injury and repair are modulated by subtype-specific SIP receptors. Whereas SIP1 expressed in proximal tubule cells attenuates kidney IRI (21), SIP3 activation initiates fibrosis in the heart (22), and SIP3 on bone marrow (BM) mesenchymal stem cells mediates fibrosis in the liver (23, 24). SIP3 regulates vascular permeability (25), arterial vasodilatation (26), and splenic endothelial sinus organization (27). In DCs, SIP3 signaling is coupled to protease activated receptor 1, leading to lethal outcomes in sepsis (28). In contrast, in some studies SIP3 is beneficial. Although activation of SIP3 protects hearts from IRI (29, 30), sphingosine 1-phosphate-3 receptor (S1pr3+) mice are protected from kidney IRI (31). Cell-specific expression of SIP3 likely contributes to the heterogeneity of outcomes. Given the importance of the DC/NKT pathway in...
kidney IRI (7) and that S1P3 is expressed on DCs (32) to regulate critical cytokines that participate in inflammation (28), we hypothesized that DC S1P3 activation of NKT cells plays an important role in kidney IRI.

Materials and Methods

All animals were handled and procedures were performed in adherence to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and all protocols were approved by the University of Virginia Institutional Animal Care and Use Committee.

Generation of BM chimeric mice

S1pr3−/− mice (a gift from Dr. Richard Proia, National Institutes of Health) and S1P3 wild-type (WT) littermate control male mice (C57BL/6 background) were used for BM transplantation as previously described (33). Briefly, lethally irradiated recipient mice were injected with 107 donor BM cells (i.v.), and the resulting chimeric mice were maintained for 6–8 wk before experimentation.

Diphertheria toxin-mediated DC ablation

CD11c-diphtheria toxin receptor (DTR) transgenic (Tg) mice (B6.FVB-Tg Ilgax-DTR/GFP 57Lan/J) harboring a transgene encoding a simian DTR–GFP fusion protein under the transcriptional control of mouse CD11c promoter were purchased (The Jackson Laboratory). DCs were depleted in male CD11c-DTR Tg mice (8 wk old, 25–28 g) by a single injection of DT (Sigma-Aldrich; 4 μg/kg body weight, i.v.), and ablation was confirmed by immunohistochemical staining for CD11c+MHCII+ DCs in kidney (6). DT-treated CD11c-DTR Tg littermates negative for the transgene (CD11c–DTR−) were used as controls. WT or S1pr3−/− BMDCs (0.5 × 105) were injected i.v. 20 h after DT injection into CD11c-DTR Tg and control mice, and IRI was performed 6 h after DC injection.

Renal IRI and blocking IL-4

Male mice (8–12 wk old, C57BL/6, from the National Cancer Institute, Frederick, MD) were subjected to bilateral IRI (24–28 min ischemia, then 24 h reperfusion) as previously described (3, 7, 12). Control, sham-operated mice underwent a similar procedure, but the renal pedicles were not clamped. Mice injected with BM-derived DCs (BMDCs) that were loaded with α-GalCer (National Institutes of Health) and S1pr3 wild-type (WT) or S1pr3−/− DCs loaded with α-GalCer or vehicle were cultured overnight with NKTL1.2 hybridoma cells (DN3A4-1-2; a gift from Dr. Kronenberg, La Jolla Institute for Allergy and Immunology) (38) at a ratio of 1:5, and changes in IL-4 cytokine levels in the cell supernatant were measured with ELISA (BD Pharmingen). As control, NKTL1.2 cells were cultured in the absence of DCs and vice versa.

S1P3 knockdown with small interfering RNA

WT DCs were seeded in medium without FBS 1 h before transfection. Two ready-to-use validated double-stranded 21-nucleotide small interfering RNAs (siRNAs) for S1P3 (J-040957-07-0010) were transfected into DCs along with ON-TARGETplus nontargeting siRNA (D-001810-01; Dharmacon, Lafayette, CA) using GenePORTER (Glenlans, San Diego, CA) and following the manufacturers’ protocols.

Activation and detection of cytokine-producing leukocytes

WT and S1pr3−/− total spleen cells or T cells isolated by negative selection from spleen (CD3+) were cultured for 72 h in supplemented RPMI 1640 under Th17-inducing conditions (2 ng/ml TGF-β, 10 ng/ml IL-23, 50 ng/ml IL-6) (all eBioscience), 2 μg/ml anti-CD3ε Ab (BD Biosciences), 1 μg/ml LPS (Sigma-Aldrich). Cells were restimulated with 1 μl/ml leukocyte activation kit (BD Biosciences) for 4–5 h and cytokine-producing cells were analyzed by flow cytometry.

Quantitative real-time RT-PCR

Total RNA was extracted from kidneys with TRI Reagent (Invitrogen) according to the manufacturer’s protocol and single-stranded cDNA was synthesized as previously described (34). Gene sequences were obtained from the GenBank database. Primers were designed using PrimerQuest (Integrated DNA Technologies, http://www.idtdna.com). Primer sequences were as previously published (12, 21). RT-PCR was performed using the iScript one-step RT-PCR kit with SYBR Green (Bio-Rad); samples were normalized to GAPDH. Melting curves were inspected to ensure specificity of product detection. The following PCR protocol was used: initial denaturation (95˚C for 3 min), denaturation, annealing, and elongation program repeated 35 times (95˚C for 45 s, 52˚C for 60 s, and 72˚C for 60 s); final elongation (72˚C for 7 min); and finally, a holding step at 4˚C.

FACS analysis

Flow cytometry was used to analyze kidney leukocyte content. In brief, kidneys were extracted, minced, digested, and passed through a filter and a cotton column as described (7). After blocking nonspecific Fc binding with anti-mouse CD16/32 (2.4G2), fresh kidney suspensions were incubated with fluorochrome-tagged anti-mouse CD45 (30-F11) to determine total leukocyte cell numbers. CD45-labeled samples were further used for labeling with different combinations of anti-mouse F4/80 (BM8), GR-1 (10B11) (BD Biosciences), 7-AAD (BD Biosciences), and CD1d tetramer loaded with α-GalCer (National Institutes of Health Tetramer Facility, Emory University, Atlanta, GA) (39). Unloaded CD1d tetramer cells were used as controls. 7-Aminoactinomycin D (7-AAD; BD Biosciences) was added 15 min before analyzing the sample to separate live from dead cells. Intracellular staining for IFN-γ was performed using the BD Biosciences (San Jose, CA) Fix/Perm buffer set according to the manufacturer’s protocol and as described previously (7, 12). Appropriate fluorochrome-conjugated, isotype-matched, irrelevant mAbs were used as negative controls. Flow cytometry data acquisition was performed on a FACSCalibur (Becton Dickinson). Data were analyzed by FlowJo software 9.0 (Tree Star). All Abs (except as noted) were from eBioscience and were used at a concentration of 5 μg/ml.

Statistical analysis

GraphPad Instat 3 (GraphPad Software), SigmaPlot 11.0 (Systat Software), and Canvas X (ACD Systems of America) were used to analyze and present

BMDC culture and NKTL1.2 hybridomas

Eight-week-old WT and S1pr3−/− male mice were used for generating highly pure DCs from whole BM precursors (35). Briefly, freshly isolated BM was cultured with 2 ng/ml recombinant mouse GM-CSF (R&D Systems) for 10 d with media changed every 3 d. Ninety-eight percent of resulting cells were CD11c+ DCs as determined by flow cytometry with CD11c and MHCII Abs. BMDCs were primed with 100 ng/ml α-GalCer or vehicle (0.1% DMSO) for 2 d in culture medium. Mouse (Vα14) and human (Vα24) invariant NKT cells can recognize α-GalCer, a glycolipid originally extracted from marine sponges (36, 37). Cells were washed, and 0.5 × 106 cells per mouse were introduced i.v. to naive mice 1 d prior to kidney IR surgery with a moderate (24 min) ischemic injury. For in vitro studies, WT or S1pr3−/− DCs loaded with α-GalCer or vehicle were cultured overnight with NKTL1.2 hybridoma cells (DN3A4-1-2; a gift from Dr. Kronenberg, La Jolla Institute for Allergy and Immunology) (38) at a ratio of 1:5, and changes in IL-4 cytokine levels in the cell supernatant were measured with ELISA (BD Pharmingen). As control, NKTL1.2 cells were cultured in the absence of DCs and vise versa.
the data. Data were analyzed, after transformation when needed to generate a normal distribution, by two-tailed t test or one- or two-way ANOVA with post hoc analysis as appropriate. A p value <0.05 was used to indicate significance.

Results
Mice deficient in BM S1P3 are protected from kidney IRI

Compared to WT mice after IRI, which have increased plasma creatinine levels indicative of reduced kidney function, S1pr3−/− mice showed no increase in creatinine (Fig. 1A), no change in infiltration of neutrophils, DCs, and macrophages (Fig. 1B), and better kidney morphology after IRI (Supplemental Fig. 1A). Chemokines and proinflammatory cytokines contribute to leukocyte infiltration following kidney IRI. WT mouse kidneys expressed more CXCL1 after IRI compared with S1pr3−/− mice; CXCL1 mRNA levels were 15.2 ± 2.7- and 0.89 ± 0.4-fold higher compared with sham in WT and S1pr3−/− mice, respectively (p < 0.01). S1pr3−/− mouse kidneys had fewer CD45+ leukocytes expressing proinflammatory cytokines IL-17 and IFN-γ and more CD45+ leukocytes and CD3+ T cells expressing anti-inflammatory cytokines IL-10 and IL-4 after IRI compared with WT mice (Fig. 1C). Additionally, the percentage of CD4+ spleen cells that express IFN-γ and IL-17 was lower in S1pr3−/− mice compared with WT mice but was comparable in CD3+ T cells isolated from spleens of WT and S1pr3−/− mice (Fig. 1D); therefore, differentiation into Th1 and Th17 cells was lower in S1pr3−/− spleens compared with WT spleens. Additionally, these data suggest that S1pr3−/− T cells can respond normally but that other leukocytes involved in the regulation of T cells are less responsive to the stimulus.

To determine the contribution of S1P3 on BM-derived cells to kidney IRI, we made BM chimeras wherein WT mice were lethally irradiated and BM was reconstituted with S1pr3−/− (S1pr3−/− → WT) or WT (WT → WT) BM cells. In a similar manner, we created a WT→S1pr3−/− chimera. After 8 wk, mice were subjected to IRI; there was a marked increase in plasma creatinine in WT→WT and WT→S1pr3−/− chimeras but not in S1pr3−/−→WT chimeras (Fig. 2A). Knees of S1pr3−/−→WT chimeras had less tubular necrosis (Fig. 2B) and lower ATN scores (Fig. 2C) after IR than did WT→WT and WT→S1pr3−/− chimeras. Kidney mRNA levels of proinflammatory cytokines and chemokines (TNF-α, IL-12p40, IL-1β, IL-6, CXCL1, CXCL2, and CXCL5) increased in WT→WT and WT→S1pr3−/− mice after IRI compared with sham but not in kidneys of S1pr3−/−→WT mice (Table I). These results indicate that S1P3 on BM-derived cells is necessary for kidney injury induced by IIR, and in its absence, the kidney is protected from IRI and there is no increase in kidney proinflammatory cytokine and chemokine expression.

Mice deficient in BM S1P3 have immature DCs in kidney after IRI and reduced infiltration of NKT cells and neutrophils compared with mice with WT BM cells

The following observations prompted further investigation of the role of BM cell S1P3 in kidney IRI: 1) key DC cytokines (TNF-α, IL-6, and IL-12p40) are regulated by S1P3 in kidney IRI (Table I) and systemic inflammation (28), 2) DCs interact with NKT cells to promote neutrophil infiltration in kidney IRI (7), and 3) S1pr3−/−→WT chimeras were protected from kidney IRI. We examined by flow cytometry the number of NKT cells (CD1d+TCRβlow), macrophages (CD11b+F4/80high), and neutrophils (CD11b+Ly6Ghigh), and the number and phenotype of DCs (CD11c+) in kidney obtained from WT→WT, S1pr3−/−→WT, and WT→S1pr3−/− mice after IRI. The total number of DCs (fold relative to sham) did not change after IRI (Table I). The total number of DCs expressing activation markers (CD11c+MHCII+), including positive costimulatory molecules (CD11c+CD40+, CD11c+CD80+, and CD11c+CD86+), increased in kidneys of WT→WT and WT→S1pr3−/− mice compared with the respective sham-operated mice, and this increase was not observed in S1pr3−/−→WT mice (Fig. 2D). Total number of CD11c+CD40+ cells did not increase in WT→S1pr3−/− mice. Activated DCs in WT→WT and WT→S1pr3−/− (but not S1pr3−/−→WT) mice after IRI also represented a larger percentage of total CD11c+ DCs (data not shown). Immunofluorescent localization revealed no obvious differences in distribution of CD11c+ cells in kidney outer medulla from any of the chimeric mice after sham or
IRI (Fig. 2F). These results demonstrate that S1P3 expressed on BM cells is important in mediating the increase in mature DCs observed in the kidney following IRI, and not simply for increased infiltration of activated DCs.

Using FACS analysis, we found an increase in the total number of infiltrating macrophages (Table I), NKT cells (Table I), and neutrophils (Fig. 2E) in kidney after IRI (consistent with our prior results in WT mice; Ref. 3) in WT→WT mice and WT→S1pr3−/− mice compared with sham, but there was no change in S1pr3−/−→WT kidneys. Confirming the flow cytometry data, immunofluorescence labeling of infiltrating neutrophils (7/4+ cells) increased in kidneys of WT→WT and WT→S1pr3−/− mice after IRI compared with sham but not in S1pr3−/−→WT chimeras (Fig. 2G). Thus, BM-derived S1P3 is necessary for neutrophil infiltration following kidney IRI. Following kidney IRI, the increase in kidney content of IFN-γ–producing leukocytes contributes to tissue inflammation (7).
Table I. Number of NKT cells, macrophages, and DCs and mRNA levels of cytokines and chemokines in kidneys of WT→WT, S1pr3−/−→WT, and WT→S1pr3−/− BM chimeras after ischemia and 24 h reperfusion

<table>
<thead>
<tr>
<th>Immune cellsa</th>
<th>WT→WT</th>
<th>S1pr3−/−→WT</th>
<th>WT→S1pr3−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>NKT cells (×10^6)</td>
<td>4.0 ± 0.84*</td>
<td>4.1 ± 1.53</td>
<td>3.8 ± 1.37</td>
</tr>
<tr>
<td>Macrophages (×10^5)</td>
<td>2.6 ± 0.49*</td>
<td>0.9 ± 0.31</td>
<td>3.1 ± 0.51*</td>
</tr>
<tr>
<td>CD11c cells (×10^5)</td>
<td>1.1 ± 0.27</td>
<td>1.7 ± 0.52</td>
<td>0.8 ± 0.24</td>
</tr>
<tr>
<td>mRNA levelsb</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>3.82 ± 0.92**</td>
<td>1.56 ± 0.56</td>
<td>4.22 ± 0.91*</td>
</tr>
<tr>
<td>IL-12p40</td>
<td>2.21 ± 0.06*</td>
<td>1.51 ± 0.16</td>
<td>2.79 ± 1.06</td>
</tr>
<tr>
<td>IL-1β</td>
<td>10.75 ± 3.35*</td>
<td>1.94 ± 0.58</td>
<td>12.46 ± 3.09**</td>
</tr>
<tr>
<td>IL-6</td>
<td>23.63 ± 6.46***</td>
<td>1.31 ± 0.35</td>
<td>12.47 ± 2.83*</td>
</tr>
<tr>
<td>CXCL1</td>
<td>31.26 ± 9.47**</td>
<td>1.23 ± 0.24</td>
<td>12.76 ± 2.23**</td>
</tr>
<tr>
<td>CXCL2</td>
<td>34.67 ± 12.15*</td>
<td>1.77 ± 0.66</td>
<td>30.68 ± 10.96**</td>
</tr>
<tr>
<td>CXCL5</td>
<td>21.92 ± 6.69**</td>
<td>4.69 ± 1.23</td>
<td>11.62 ± 3.61**</td>
</tr>
</tbody>
</table>

*For immune cells, the total numbers of NKT (CD45^-AAD CD19^ TCR^low^CD4^-CD8^-) cells, macrophages (CD45^-AAD CD11b^-F4/80^-), and CD11c (CD45^-AAD CD11c^-) cells, expressed as fold ratios relative to respective sham-operated mice and as determined by flow cytometry, are shown (n = 3–6/group). Data are means ± SEM. *p < 0.05 relative to sham-operated mice.

b mRNA expression levels shown are relative to GAPDH and are expressed as fold ratios relative to respective sham-operated mice (n = 4–6/group). Data are means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 relative to sham-operated mice.

We next sought to determine whether S1P3 on BM-derived cells regulates IFN-γ production after kidney IRI. There was a significant increase in the total number of IFN-γ-producing leukocytes (CD3^-T cells and CD3^-non-T cells) (Fig. 2H) in kidney in WT→WT and WT→S1pr3−/− mice after IRI and in IFN-γ content in CD3^- and CD3^-cell populations (Fig. 2I), but this increase was not observed in S1pr3−/−→WT mice. S1P3-deficient DCs attenuate kidney IRI

Following IRI, key DC cytokines (TNF-α, IL-12p40, and IL-1β) from S1pr3−/−→WT mouse kidneys were attenuated compared with control chimeras, suggesting that the lack of immune response in mice with S1P3-deficient BM could be due to S1P3-deficient DCs. To demonstrate the functional role of DCs expressing S1P3 in kidney IRI, we performed adoptive transfer studies in mice depleted of DCs. First, the optimal number of DCs for these reconstitution studies was determined. Mild ischemia alone (no DCs) caused only a minimal rise in plasma creatinine compared with sham-operated mice (dashed line), but injection of WT DCs exacerbated injury in a dose-dependent manner as indicated by increased plasma creatinine (Fig. 3A), kidney histology (Supplemental Fig. 1B), and tubular injury (data not shown). A dose of 0.5 × 10^5 DCs, which did not exacerbate injury in intact WT mice, was selected for adoptive transfer studies. DCs were depleted in DT-sensitive recipient mice that express the silim antigen DTR under the control of the CD11c promoter (CD11c-DTR); littersmates lacking DTR transgene expression (CD11c^−/−) were used as controls. Depletion of DCs by DT administration to CD11c-DTR^+ mice 24 h prior to kidney IR reduced kidney DC content (6) and in the present study led to a marked attenuation of kidney injury (as measured by plasma creatinine) (Fig. 3B) and kidney histology (Supplemental Fig. 1C). Kidney mRNA levels of proinflammatory cytokines and chemokines increased markedly in WT mice after IRI, but TNF-α (1.08 ± 0.39), IL-12p40 (0.19 ± 0.49), IL-1β (0.47 ± 0.68), IL-6 (1.64 ± 0.76), CXCL1 (0.48 ± 0.45), CXCL2 (5.63 ± 0.32), and CXCL5 (4.68 ± 0.67) (fold expression relative to sham) did not change significantly after IRI in DC-depleted mice, demonstrating that DCs are necessary to initiate the inflammatory immune response after kidney IRI. The reduction in injury in DC-depleted mice was reversed by adoptive transfer of WT but not S1pr3−/− BMDCs (Fig. 3B–D). Kidney neutrophil infiltration, a hallmark of IR, which increased only modestly after IR in DC-depleted CD11c-DTR^+ mice relative to sham, was higher after adoptive transfer of WT but not S1pr3−/− DCs (Fig. 3D). These results indicate that DC S1P3 is necessary in mediating injury following IR. Additionally, protection from injury is not due to compensatory changes in expression of the other S1P receptor subtypes in DCs of S1pr3−/− mice, as expression was different from WT DCs (mRNA by RT-PCR, data not shown).

DCs require S1P3 to initiate inflammation following IRI

DC-mediated activation of NKT cells depends on stimulation of the invariant TCR through glycolipid presentation, costimulation, and cytokine production. To further explore the role of DC S1P3 in injury, we investigated whether S1P3 is necessary for DCs to activate NKT cells. CD1d expressed by DCs presents either self-glycolipid, such as isoglobotrihexosylceramide (40), or in experimental protocols, foreign glycolipid, such as the marine sponge-derived α-GalCer (41), to NKT cells. Following a widely used experimental paradigm to probe the role of NKT cell activation, BMDCs from WT and S1pr3−/− mice were loaded with vehicle (DMSO) or α-GalCer and were administered (i.v.) 24 h before a subthreshold "mild" kidney IRI in mice. We used a mild injury model to detect the anticipated increase in injury following activation of NKT cells by α-GalCer–loaded DCs. Consistent with prior findings that DC-α-GalCer–mediated NKT cell activation exacerbates kidney IRI (12), WT mice that received α-GalCer–loaded WT DCs showed significant functional (Fig. 4A) and morphological (Supplemental Fig. 1D) evidence of injury; injury was not elicited by α-GalCer–loaded S1pr3−/− DCs. Similarly, mRNA expression of proinflammatory cytokines and chemokines (TNF-α, IL-12p40, IL-1β, IL-6, CXCL1, CXCL2, and CXCL5) increased after IR compared with sham in mice that received α-GalCer–loaded WT but not α-GalCer–loaded S1pr3−/− DCs (Table II). Mild ischemia did not increase infiltration of NKT cells and neutrophils; however, increased cell infiltration produced by α-GalCer–loaded WT DCs in sham-operated mice was further enhanced by IR, particularly for neutrophil infiltration. In contrast, α-GalCer–loaded S1pr3−/− DCs did not stimulate neutrophil infiltration after IR (Fig. 4B). These results indicate that DCs harboring S1P3 are necessary for activation of NKT cells and downstream infiltration of neutrophils in kidney IRI. Furthermore, maturation of DCs, which can be induced by α-GalCer–activated NKT cells (42), also required DC S1P3. The subthreshold injury did not increase the number of mature DCs (data not shown), which was produced by our standard 28 min ischemia (Fig. 2D), but administration of α-GalCer–loaded WT DCs increased sub-
substantially the total CD11c<sup>+</sup>MHCII<sup>+</sup>, CD11c<sup>+</sup>CD40<sup>+</sup>, and CD11c<sup>+</sup>CD1d<sup>+</sup> cells observed following mild IRI (Table II). In contrast, there was no enhancement of these populations of activated DCs when α-GalCer–loaded S1pr<sup>−/−</sup> DCs were injected into mice prior to IRI. Therefore, not only are DCs expressing S1P3 necessary for activating NKT cells in IRI, but the accompanying DC maturation may be mediated by the S1P3-dependent DC–α-GalCer–stimulated activation of NKT cells.

**DCs deficient of S1P3 induce a Th2-like response in NKT cells**

To determine whether S1P3 is important for cytokine production following DC-mediated activation of NKT cells, WT and S1pr<sup>−/−</sup> mice were administered α-GalCer (10 μg/mouse, i.p.), and plasma IFN-γ levels were measured over time. NKT cells activated by this process rapidly release large amounts of both IL-4 and IFN-γ (43, 44). Plasma IFN-γ levels were higher in WT mice 6 h after treatment with α-GalCer in WT mice (and were sustained for up to 24 h) but did not increase in S1pr<sup>−/−</sup> mice (Fig. 5A). Conversely, IL-4 plasma levels were higher in S1pr<sup>−/−</sup> mice than in WT mice 4 h after α-GalCer administration. Liver NKT cell–dependent production of IL-4 and IFN-γ was measured by FACS 2 h after α-GalCer (1 μg/mouse, i.v.) as previously described (38) without restimulation. More liver NKT cells (CD1<sup>−</sup>ε<sup>+</sup>-positive cells) from WT mice made IFN-γ than did NKT cells from S1pr<sup>−/−</sup> mice, but more S1pr<sup>−/−</sup> NKT cells made IL-4 than did WT NKT cells (Fig. 5B). To directly test the interaction of WT and S1pr<sup>−/−</sup> DCs with NKT cells we cultured vehicle- and α-GalCer–loaded DCs with NKT1.2 hybridoma cells, which produce IL-4 but not IFN-γ upon stimulation with α-GalCer–loaded DCs (38). Higher levels of IL-4 were found after coculture with α-GalCer–loaded S1pr<sup>−/−</sup> DCs than with vehicle-loaded S1pr<sup>−/−</sup> DCs or α-GalCer–loaded WT DCs (Fig. 5C). These data suggest that DCs deficient of S1P3 induce a Th2-like response in NKT cells to produce more IL-4, although it is possible in vivo that conventional CD4<sup>+</sup> T cells could contribute to increased plasma IL-4 levels and hence to the Th2 response.

**Protection of S1pr<sup>−/−</sup> mice from IRI is IL-4–dependent**

Our in vitro studies demonstrated that S1P3 deficiency in DCs leads to Th2 polarization with production of IL-4. Therefore to determine in vivo whether the protection observed in S1P3-deficient mice was IL-4–dependent, we injected WT and S1pr<sup>−/−</sup> mice with a mAb to IL-4 (IL-4 mAb) 1 d prior to IR to block the effects of IL-4. IL-4 mAb had no effect on injury in WT mice but reversed the protection from injury in S1pr<sup>−/−</sup> mice in a dose-dependent manner compared with isotype control (IgG1)-injected S1pr<sup>−/−</sup> mice as measured by plasma creatinine (Fig. 5D) or kidney neutrophil infiltration (Fig. 5E). WT or S1pr<sup>−/−</sup> mice injected with 100 μg IL-4 mAb died several hours after surgery (data not shown). Taken together these data suggest that the protection observed in S1pr<sup>−/−</sup> mice was IL-4–dependent.

**WT DCs reconstitute injury in S1pr<sup>−/−</sup> mice**

We next sought to determine whether S1P3 deficiency on non-DCs conferred kidney protection following IRI. In this experiment WT DCs were adoptively transferred into S1pr<sup>−/−</sup> mice (containing S1pr<sup>−/−</sup> NKT cells). S1pr<sup>−/−</sup> mice were protected from severe IRI (28 min ischemia). Injury was reconstituted in S1pr<sup>−/−</sup> mice injected with 0.5 × 10<sup>6</sup> WT DCs but not with 0.5 × 10<sup>6</sup> S1pr<sup>−/−</sup> DCs.
DCs (Fig. 6A). After IRI, S1pr3−/− mice also had significantly more tubular injury (Supplemental Fig. 1E) and neutrophil infiltration (Fig. 6B) after injection of WT DCs than after S1pr3−/− DCs, thus demonstrating that DC S1P3 is required for kidney injury. Additionally, non-DCs, including NKT cells, deficient of S1P3 do not confer kidney protection from IRI.

To further demonstrate there was no defect in the T cell or NKT cell response in S1pr3−/− mice, we isolated conventional CD4+ T cells (CD45+7-AAD−CD11c−CD1d−) from WT and S1pr3−/− mice. Equal numbers of conventional T cells were injected into Rag-1−/− mice a week before kidney IRI. Reconstitution of T and B cell-deficient Rag-1−/− mice, which are resistant to injury, with WT or S1pr3−/− conventional T cells resulted in a similar rise in plasma creatinine, suggesting that Tcon cells from S1pr3−/− mice had no defect in their ability to contribute to the inflammatory response to IRI (Fig. 6C). These results demonstrate that NKT and T cells recognize and respond to DC-presented α-GalCer and are activated in IRI in the absence of NKT cell S1P3.

Table II. Numbers of NKT cells and activated DCs (CD11c+) and mRNA levels of cytokines and chemokines in kidney after 24 h reperfusion from sham and ischemic mice injected with WT or S1pr3−/− BMDCs loaded with vehicle or α-GalCer

<table>
<thead>
<tr>
<th>Immune cells*</th>
<th>WT DC</th>
<th>WT DC-α-GalCer</th>
<th>S1pr3−/− DC</th>
<th>S1pr3−/− DC-α-GalCer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>IRI</td>
<td>Sham</td>
<td>IRI</td>
</tr>
<tr>
<td>NKT (×105)</td>
<td>1.9 ± 0.5</td>
<td>2.2 ± 0.4</td>
<td>9.7 ± 0.6</td>
<td>15.1 ± 2.2***</td>
</tr>
<tr>
<td>CD11c-MHCII* (×105)</td>
<td>9.7 ± 3.4</td>
<td>38.4 ± 6.4</td>
<td>23.0 ± 84.5</td>
<td>1049.5 ± 294***</td>
</tr>
<tr>
<td>CD11c-CD40* (×105)</td>
<td>1.1 ± 0.2</td>
<td>1.5 ± 0.5</td>
<td>19.2 ± 66.1</td>
<td>86.9 ± 23.8**</td>
</tr>
<tr>
<td>CD11c-CD1d* (×105)</td>
<td>0.4 ± 0.1</td>
<td>1.7 ± 0.5</td>
<td>55.9 ± 29.3</td>
<td>515.5 ± 125.9***</td>
</tr>
<tr>
<td>mRNA levels**</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>4.16 ± 0.67</td>
<td>1.04 ± 0.21</td>
<td>4.13 ± 0.64</td>
<td>0.74 ± 0.15</td>
</tr>
<tr>
<td>IL-12p40</td>
<td>3.24 ± 1.46</td>
<td>0.52 ± 0.14</td>
<td>0.22 ± 0.10</td>
<td>0.93 ± 0.67</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.89 ± 0.40</td>
<td>3.17 ± 0.71**</td>
<td>2.75 ± 0.67</td>
<td>0.69 ± 0.32</td>
</tr>
<tr>
<td>IL-6</td>
<td>10.21 ± 3.99</td>
<td>52.2 ± 82.81***</td>
<td>18.1 ± 9.35</td>
<td>0.46 ± 0.24</td>
</tr>
<tr>
<td>CXCL1</td>
<td>11.42 ± 3.56</td>
<td>18.63 ± 0.59</td>
<td>12.66 ± 1.37</td>
<td>1.52 ± 0.46</td>
</tr>
<tr>
<td>CXCL2</td>
<td>10.94 ± 4.86</td>
<td>84.45 ± 6.48**</td>
<td>2.82 ± 0.51</td>
<td>1.51 ± 0.21</td>
</tr>
<tr>
<td>CXCL5</td>
<td>35.07 ± 13.43*</td>
<td>61.40 ± 9.04*</td>
<td>37.69 ± 15.02*</td>
<td>6.32 ± 2.59***</td>
</tr>
</tbody>
</table>

*For immune cells, total numbers of NKT (CD45+7-AAD−CD19 TCR6+CD1d−) and CD11c (CD45+7-AAD−CD40+CD11c+) cells as determined by flow cytometry are shown (n = 3–6/group). Data are means ± SEM. **p < 0.01, ***p < 0.001 compared with WT DC IRI; †p < 0.05, ††p < 0.001 compared with WT DC-α-GalCer IRI.

**mRNA expression levels are shown relative to GAPDH and are expressed as fold ratios relative to respective sham-operated mice (n = 4–6/group). Data are means ± SEM.

*p < 0.05, **p < 0.01, ***p < 0.001 relative to sham-operated mice.
Prevention of acute kidney injury and treatment of established acute kidney injury

Our data strongly suggested that S1P3 controls DC phenotype and its absence renders DCs immature and tolerized. Therefore, we investigated the potential clinical application and efficacy in prevention of IRI by administering S1pr3−/− mice 7 d prior to IRI. As in the results with 2 d pretreatment, 7 d pretreatment with α-GalCer–loaded S1pr3−/− DCs resulted in significantly less injury (reduced plasma creatinine; Fig. 7A), neutrophil infiltration (Fig. 7B), and tubular injury (by H&E; Supplemental Fig. 1F) after kidney IRI compared with pretreatment with WT DCs or α-GalCer–loaded WT DCs. Next, we sought to determine the effectiveness of this approach in treating established injury following IR. Mice were significantly protected from kidney injury when administration of α-GalCer–loaded S1pr3−/− DCs was delayed by 3 h after IR (Fig. 7C) and tubular injury score (Fig. 7D, by H&E; Supplemental Fig. 1G) compared with saline or WT DC–α-GalCer.

Confirmation of results with Tg mice on the role of DC S1P3 in IRI was obtained in siRNA experiments. Knockdown of S1P3 expression in WT DCs using siRNA (WT-siRNA) prior to loading with α-GalCer and injection into WT mice resulted in less injury (Fig. 7E) and less neutrophil infiltration (by flow cytometry) (E) were measured after 20 h of reperfusion (n = 3/group). Data are expressed as means ± SEM. *p < 0.05, **p < 0.01.
**Discussion**

SIP is an important intracellular and extracellular signaling molecule that regulates cellular function and modulates the immune system. In this study, we defined a permissive role of SIP3 in the DC response to kidney IRI and in DC-mediated activation of NKT cells and neutrophil infiltration (Fig. 8). We found that DC SIP3 plays an important role in mediating kidney IRI, and its absence from DCs attenuated kidney injury. The absence of SIP3 blocked DC maturation, as evidenced by reduced expression of activated NKT cells, and do not induce immune cell infiltration; the marked increase in expression of macrophage- and DC-associated proinflammatory genes normally observed after IRI in kidneys was also absent. Furthermore, although SIP3 on DCs is necessary to activate the DC/NKT pathway that is important for initiating kidney injury, our data suggest that T cell SIP3 does not appear to be critical for this response, as T cells, and specifically NKT cells, from SIP3−/− mice function normally in response to a stimulus or in contributing to IRI.

Mouse NKT cells express the invariant TCR, Vα14Jα18, and human NKT cells express an invariant Vα24 TCR (57). Both mouse and human NKT cells are dependent on CD1d for positive selection in the thymus and subsequent activation in the periphery (10, 58). DC-mediated presentation of endogenous self-glycolipid or α-GalCer via CD1d activates NKT cells, which results in IFN-γ production following reperfusion (7), but in the present study the number of CD45+ IFN-γ cells did not increase after IRI in SIP3-deficient mice. These findings provide further evidence for a role...
FIGURE 7. S1pr3−/− DCs are effective both in preventing kidney IRI and in treating injury after IR. (A and B) WT mice were injected (i.v.) with WT DCs or S1pr3−/− DCs (0.5 × 10^6; loaded with α-GalCer) 7 d prior to IRI (26 min). (A) Plasma creatinine; (B) neutrophil infiltration in kidneys (n = 3–4/group). *p < 0.05, **p < 0.01, ***p < 0.001. (C) WT mice were subjected to 26 min ischemia and 24 h reperfusion and injected (i.v.) with WT DCs or S1pr3−/− DCs (0.5 × 10^6; loaded with α-GalCer) 3 h after ischemia; plasma creatinine was measured at the 24 h reperfusion. Dashed line, creatinine levels in sham-operated mice (n = 3–4, p < 0.001). (D) Semiquantitative measure of tubular injury in H&E-stained kidney sections. Dashed line, ATN in sham-operated mice. (E) Knockdown of S1pr3 in WT DCs results in less kidney injury in WT mice as indicated by plasma creatinine and (F) neutrophil infiltration. WT DCs transfected with S1P3 siRNA (WT-siRNA) or scrambled oligonucleotides (WT-Scr) or untreated WT or S1pr3−/− DCs were loaded with α-GalCer and injected into WT mice (0.5 × 10^6 cells) 24 h prior to IRI (n = 3–4/group). Data are means ± SEM. *p < 0.05, **p < 0.01.
deficient of S1P3 have reduced migration and endocytosis in response to S1P (69). Although it is possible that reduced DC migration contributes to the lack of injury in the absence of DC S1P3 in our kidney IR model, we did not observe any differences in DC migration in WT and S1pr3−/− mice using FITC skin painting and footpad injection of DCs treated with LPS (A. Bajwa and M.D. Okusa, unpublished observations). Furthermore, no significant differences in accumulation of i.v. injected DCs were observed between WT and S1pr3−/− DCs. Injection of unloaded or α-GalCer-loaded WT or S1pr3−/− DCs into CD45.1 mice resulted in accumulation of injected DCs in lymphoid and nonlymphoid organs, including spleen, liver, lung, and peripheral lymph nodes (data not shown); these results are similar to previously published findings (71). Thus, the effects of transferred DCs on IRI may be mediated at distant sites; identification of a mechanism will require further study.

A current strategy for reducing the incidence of rejection in transplantation is to produce immature DCs with a stable tolerogenic phenotype (72–79) using various pharmacological agents (80) or to deplete donor MHCII+ or CD45+ passenger leukocytes with mAbs prior to transplantation (81). Similarly, S1pr3−/− DCs administered after established IRI have not yet been identified and could include enhanced repair processes.

A

![Diagram A](Image 151x600 to 407x732)

**FIGURE 8.** Absence of S1P3 in DCs polarizes T cells to a Th2 phenotype in kidney IRI. In kidney IRI, (A) WT DCs via their class I-like CD1d molecule present endogenous glycolipid or α-GalCer to NKT cells and along with CD40 costimulatory molecule–CD40L interaction cause NKT cell activation. Additionally, DCs can interact with and activate conventional T cells and regulatory T cells through a variety of mechanisms. Activated NKT cells produce large amounts of IFN-γ (Th1 response), leading to neutrophil infiltration and kidney injury. (B) DCs lacking S1P3 (S1pr3−/− DCs) also present endogenous glycolipid or α-GalCer to NKT cells via their class I-like CD1d molecule but have reduced CD40 and cytokine/chemokine expression after kidney IRI. NKT cells stimulated by α-GalCer-loaded S1pr3−/− DCs produce large amounts of IL-4 (Th2 response) and IL-10 with low to minimal IFN-γ (Th1) and IL-17. Similarly, S1pr3−/− DCs in mice subject to kidney IRI may fail to induce a Th1 response in conventional T cells and hence promote increased IL-4 production by conventional T cells. High levels of IL-4 result in less neutrophil infiltration and less kidney injury. Neutralization of IL-4 with blocking mAb reverses this protective effect of IL-4 in S1pr3−/− mice, leading to more kidney injury. The present studies focused on DC/NKT interactions in IRI, but other mechanisms, such as reduced IL-17 or increased IL-10, may also contribute to protection in S1pr3−/− mice and in WT mice treated with S1pr3−/− DCs prior to IRI. Mechanisms underlying the beneficial effects of S1pr3−/− DCs administered after established IRI have not yet been identified and could include enhanced repair processes.
a rationale for development of a selective S1P3 antagonist, particularly in view of the absence of safe and effective Food and Drug Administration-approved drugs for the treatment of acute kidney injury. In contrast to our results, Park et al. (68) demonstrated that a commercially available S1P3 antagonist (CAY10444) did not provide protection from kidney IRI, but the dose and timing of administration may not have been optimal for blocking S1P3, and the specificity and efficacy of this compound at S1P3 has been questioned (90). Thus, the development of a potent and selective compound is necessary for future studies.

In summary, the present study demonstrates a role for sphingolipid S1P3 on DCs in kidney IRI. S1P3 is necessary for DCs to: (1) exhibit a mature phenotype, (2) regulate IFN-γ in the innate immune response to injury, and (3) regulate the innate immune response and NKT cell and neutrophil transmigration to the injured kidney following kidney IRI. S1P3 controls NKT Th1–Th2 polarity: the absence of S1P3 leads to a Th2 phenotype and the presence of S1P3 leads to a Th1 phenotype. Our studies, however, do not exclude other potential mechanisms of DC S1P3 in the control of innate immune response to IRI, and further identification of the role of S1P3 on DCs will add to our understanding of the immunological pathways involved in kidney IRI. These studies should aid in development of new therapeutic strategies for patients with acute kidney injury.

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Disclosures

The authors have no financial conflicts of interest.

References


Corrections


An additional funding source has been added to the grant footnote. The corrected grant footnote is shown below.

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