Hypoxia-Inducible Factor-2α Limits Natural Killer T Cell Cytotoxicity in Renal Ischemia/Reperfusion Injury

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ABSTRACT

Natural killer T (NKT) cells are the major early-acting immune cell type and fundamental immune modulators in ischemia-reperfusion injury (IRI). Because lymphocytes are exposed to various oxygen tensions under pathophysiologic conditions, we hypothesize that hypoxia-inducible factors (HIFs) have roles in NKT cell activation, and thus determine the final outcome of renal IRI. In this study, we used Lck-Cre transgenic mice to specifically disrupt HIF-2α in T/NKT cells and found that HIF-2α knockout led to upregulated Fas ligand expression on peripheral NKT cells, but not on conventional T cells. HIF-2α knockout promoted infiltration of NKT cells into ischemic kidneys and exacerbated IRI, which could be mitigated by in vivo NK1.1+ cell depletion or Fas ligand blockade. Compared with wild-type NKT cells, HIF-2α−/− NKT cells adoptively transferred to Rag1-knockout mice elicited more severe renal injury, and these mice were not protected by CGS21680, an adenosine A2A receptor agonist. Mechanistically, hypoxia-induced expression of adenosine A2A receptor in NKT cells and CGS21680-induced cAMP production in thymocytes were HIF-2α-dependent. Hydrogen peroxide-induced Fas ligand expression on thymic wild-type NKT cells was significantly attenuated by CGS21680 treatment, but this effect was lost in HIF-2α−/− NKT cells. Finally, CGS21680 and LPS, an inducer of HIF-2α in endothelium, synergistically reduced renal IRI substantially, but this effect was absent in Mx1-Cre-induced global HIF-2α-knockout mice. Taken together, our results reveal a hypoxia/HIF-2α/adenosine A2A receptor axis that restricts NKT cell activation when confronted with oxidative stress and thus protects against renal IRI.


Renal ischemia/reperfusion injury (IRI) has important implications in clinical transplantation. IRI is a multifactorial process, among which the inflammatory response is an important contributor.1,2 CD4+ T cell is a key player in the pathogenesis of renal IRI.3,4 T cell deficiency results in resistance to renal IRI, which can be restored by the adoptive transfer of CD4+ T cells.5,6 CD4+ T cells consist of functionally distinct subsets including conventional T cells and natural killer T (NKT) cells. In contrast to T cells, NKT cells respond to stress within hours, which makes them an ideal candidate to participate in the early immune response to IR. Previous studies7,8 revealed NKT cells to be the major early-acting CD4+ cell type in IRI, which regulated the function of other subsets of inflammatory cells, and thus served as a fundamental immune modulator in IRI.

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Lymphocytes frequently encounter a wide range of oxygen tensions given their highly mobile nature, and T/NK T cell responses in the context of inflammation are influenced by both hypoxia exposure and the expression of hypoxia-inducible factors (HIF), which are transcription factors regulating oxygen homeostasis. It has been shown that HIF-1α plays crucial roles in T cell survival and functions. However, the role of HIF-2α in T/NKT cells has not been explored.

HIF-2α shares 48% identity with HIF-1α and is an important transcriptional regulator of hypoxic responses, controlling a variety of processes including EPO synthesis, lipid metabolism, iron homeostasis, vascular tumorigenesis, and macrophage function. Studies from our group and others have demonstrated that HIF-2α plays a key protective role in renal IRI, by preserving endothelial integrity and functions. However, whether HIF-2α in T/NKT cells plays a role in renal IRI still remains to be elucidated.

In this study, we crossed the Lck-Cre mice with HIF-2α floxed mice and generated Lck-Cre+/HIF-2αfl/fl mice and generated Lck-Cre+HIF-2αfllox/fllox (HIF-2α−/−) mice, to explore the role of T/NKT cell HIF-2α in renal IRI. Our results demonstrated that HIF-2α was very important in limiting NKT cell cytotoxicity in renal IRI.

RESULTS

HIF-2α Knockout Led to Upregulated FasL Expression on Peripheral NKT Cells, but not on Conventional T Cells

Various stress stimuli induce FasL expression on T/NKT cells and the in vivo cytotoxicity of NKT cells depends mainly on the Fas/FasL interaction. It was reported that blockade of the Fas/FasL interaction attenuated IRI both in the kidney and in the liver. So we isolated thymocytes, liver mononuclear cells (MNCs) and splenocytes, which were subjected to flow cytometry analyses. The results are summarized in Table 1, and a typical result is shown in Figure 1. Compared with the wild-type (WT) counterpart, HIF-2α−/− mice had relatively less NKT/T cells in the spleen and liver (but not in the thymus). NKT cells from HIF-2α−/− livers and spleens were characterized by much higher FasL expression. By contrast, FasL expression on conventional T cells or NK cells was not influenced by HIF-2α knockout (KO). Studies employing Mx1-Cre+HIF-2αfllox/fllox mice (overall HIF-2α KO) showed consistent results (Supplemental Figure 1).

Lck-Cre-Mediated HIF-2α KO Exacerbated Renal IRI by Promoting Infiltration of CD4+ NKT Cells into Ischemic Kidneys

HIF-2α−/− and WT mice were subjected to renal IRI procedures. At 24 hours after reperfusion, the serum levels of creatinine (Cr) and BUN were evaluated and the results are shown in Figure 2, A and B. In the groups of 20 minutes of ischemia, HIF-2α−/− mice exhibited a significant increase in both Cr and BUN levels, suggesting exacerbated renal dysfunction. There was no statistical difference in the groups of 25 min of ischemia, probably because the renal damage had reached the highest limit, which equaled the level of mice subjected to bilateral nephrectomy (data not shown). Separate groups of mice were subjected to survival experiments. All WT mice survived the challenge of 20 minutes of ischemia. By contrast, only 30% HIF-2α−/− mice could survive (P<0.05, Figure 2C). The observations were reinforced by histologic evidence (Figure 2D).

Renal sections were then subjected to immunohistofluorescence staining. As shown in Figure 3A and Table 2, the relatively mild ischemic challenge (20 minutes) promoted infiltration of few WT NKT cells into the ischemic kidneys at 3 hours after reperfusion. However, much more HIF-2α−/− NKT cells infiltrated into the kidneys at 3 hours and thereafter. In both groups, most infiltrating CD4+ cells were also NK1.1+, showing that NKT cells rather than conventional T cells dominated the immune responses. These observations were consistent with results from flow

| Table 1. Proportion of lymphocyte subset and percentage of FasL+ cells in liver MNCs, splenocytes and thymocytes harvested from HIF-2α−/− mice and WT littermates |
|------------------|------------------|------------------|
|                  | NK1.1+TCRβ+      | NK1.1−TCRβ+      | NK1.1+TCRβ+      |
| NK1.1+TCRβ* liver MNCs | 5.8±1.3*         | 21.8±4.2*        | 15.7±4.3         |
| WT liver MNCs     | 11.2±2.7         | 37.7±5.7         | 17.6±5.1         |
| HIF-2α−/− spleenocytes | 1.1±0.3*         | 31.2±4.8*        | 8.9±2.5          |
| WT spleenocytes   | 1.9±0.4          | 45.9±6.8         | 7.9±2.6          |
| HIF-2α−/− thymocytes | 0.66±0.11        | 13.6±3.4         |                  |
| WT thymocytes     | 0.65±0.13        | 14.1±3.1         |                  |
| % FasL+ cells (mean±SD) |                  |                  |                  |
| HIF-2α−/− liver MNCs | 26.6±10.9*       | 4.6±1.3          | 3.3±1.2          |
| WT liver MNCs     | 6.8±2.1          | 4.9±1.6          | 3.5±1.6          |
| HIF-2α−/− spleenocytes | 22.8±8.6*        | 0.26±0.11        | 0.89±0.24        |
| WT spleenocytes   | 4.5±1.4          | 0.21±0.12        | 0.88±0.32        |
| HIF-2α−/− thymocytes | 2.3±0.9          | 0.79±0.24        |                  |
| WT thymocytes     | 2.2±1.1          | 0.76±0.23        |                  |

Values represent mean±SD of six mice of each group.

*P<0.05 by t test compared with the values of WT controls, respectively.
Figure 1. Proportion of lymphocyte subset and percentage of FasL+ cells in liver MNCs, splenocytes and thymocytes harvested from HIF-2α−/− mice and WT littermates. The mice received no previous treatments before the lymphoid organs were harvested. Single-cell suspensions were prepared as described in the Methods, and then stained with antibodies against NK1.1 (APC), TCR-β (FITC) and FasL (PE). Expression of FasL was analyzed on electronically gated NK1.1+TCR-β+(NKT), NK1.1−TCR-β+(T), or NK1.1+TCR-β−(NK) cells. Blue lines indicate the staining of FasL antibody, and red lines indicate the background staining with isotype-matched control IgG. Similar results were obtained in six independent experiments.
cytometry analysis of the renal inflammatory cells isolated at 3 hours after reperfusion. Moreover, the infiltrating NKT cells were fully activated because they were all FasL+ (Figure 3B).

These results suggested that HIF-2α−/− NKT cells responded much more actively and quickly to ischemic stress, and might be responsible for the exacerbated renal IRI.
Figure 3. HIF-2α KO promoted infiltration of NKT cells into ischemic kidneys. (A) HIF-2α−/− and WT mice were subjected to 20 minutes of renal ischemia. Renal sections from sham-operated mice or ischemic kidneys harvested at 3, 6, and 24 hours after ischemic insult were stained with the indicated antibodies, followed by confocal microscopic analyses. Representative photographs are shown. Original magnification, ×200. Similar results were obtained in six independent experiments and summarized in Table 2. (B) HIF-2α−/− mice and WT
As shown in Figure 5E, FasL Ab exacerbated renal IRI in HIF-2α–/– mice, indicating that HIF-2α was necessary for the anti-inflammatory effect of adora2a activation. Consistently, CGS21680 also lost effect in Mx1-HIF-2α−/− mice (data not shown).

Compared with WT NKT Cells, Adoptive Transfer of HIF-2α−/− NKT Cells Elicited more severe Renal IRI to Rag1KO Mice, which was Resistant to CGS21680 Treatment

Cre recombinase specifically targeting NKT cells is not yet available, so we conducted experiments involving the adoptive transfer of NKT cells to congenic Rag1 KO (Rag1KO on C57B/6 background) mice that lacked lymphocytes including T/NKT cells.24 Using NK1.1+ iNKT Cell isolation kit, we isolated NKT lymphocytes from spleens, which were adoptively transferred into Rag1KO mice at 4 d before renal IR. Although both WT and HIF-2α−/− NKT cells reconstituted renal IR injury, the latter induced more severe damage. CGS21680 protected against renal damage when WT but not HIF-2α−/− NKT cells were transferred. The results of serum creatinine (Figure 7A) and BUN (data not shown) were consistent with periodic acid–Schiff-stained renal sections (Figure 7B). These findings suggest that HIF-2α was necessary to limit NKT cell cytotoxicity in renal IRI, probably by regulating adora2a expression and function in NKT cells.

Hypoxia-Induced Adora2a Expression in NKT Cells was HIF-2α-Dependent

T cell receptor (TCR)-triggered FasL upregulation in T lymphocytes could be suppressed by adora2a activation.27,28 To determine whether adora2a was behind FasL upregulation in HIF-2α−/− NKT cells and the role of HIF-2α in adora2a expression, WT and Mx1-HIF-2α−/− mice were placed in air-tight chambers under normoxic (21% oxygen) or hypoxic conditions (10% oxygen). As shown in Figure 8, A and B, adora2a was constitutively expressed in thymocytes but not splenocytes. Hypoxia induced adora2a expression in splenocytes. However, adora2a could hardly be detected in Mx1-HIF-2α−/− thymuses or spleens. Splenocytes were then subjected to flow cytometry analyses, which showed that hypoxia treatment upregulated adora2a expression on WT NKT cells, which was almost absent in HIF-2α−/− NKT cells (Figure 8C). HIF-2 was previously shown to bind with high affinity to the promoter of adora2a.29 However, whether this was true in thymocytes has not been investigated previously. HIF binding activity to adora2a promoter was determined by electrophoretic mobility shift assay (EMSA) analysis using nuclear

Table 2. Number of CD4+ cells/high power field in post-ischemic kidneys harvested from HIF-2α−/− mice and WT littermates

<table>
<thead>
<tr>
<th>Sham3h</th>
<th>IR3h+</th>
<th>IR6h+</th>
<th>IR9h+</th>
<th>IR12h+</th>
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<td>WT</td>
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<td>56±17</td>
<td>278±66</td>
<td>298±48</td>
<td>271±87</td>
</tr>
<tr>
<td>HIF-2α−/−</td>
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<td>530±97</td>
<td>468±78</td>
<td>436±54</td>
<td>387±68</td>
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</tbody>
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Values represent mean±SD of four mice of each group. *P<0.05 by t test between the two groups.

In Vivo NK1.1+ Cell Depletion or FasL Blockade Reduced Renal IRI and Eliminated the Difference between HIF-2α−/− and WT Mice

Because Lck-Cre recombinase led to gene KO in both conventional T cells (NK1.1+) and NKT cells (NK1.1+), to test the hypothesis that NKTrather than T cells contributed to the exacerbated IRI in HIF-2α−/− mice, we employed a monoclonal anti-NK1.1 antibody PK136.6 Treatment with PK136 substantially depleted NK1.1+ cells in the thymocytes, liver MNCs, and splenocytes as assessed by FACS analysis while leaving conventional T cell number intact (Figure 4A). Depletion of NK1.1+ cells reduced renal damage. More importantly, PK136 eliminated the difference between HIF-2α−/− and WT mice, and led to indefinite survival in all HIF-2α−/− mice (Figure 4, B–D). Because Lck-Cre-mediated gene targeting did not involve NK cells, these results suggested that NKT cells were responsible for the exacerbated renal IRI in HIF-2α−/− mice.

As stated above, upregulated FasL expression was observed on HIF-2α−/− NKT cells, which infiltrated into post-ischemic kidneys aggressively. To determine the role of FasL in the exacerbated renal IRI in HIF-2α−/− mice, post-ischemic kidneys were subjected to immunoblotting analysis and a representative result is shown in Figure 5A, which revealed much higher FasL expressions in HIF-2α−/− kidneys. We next employed a FasL blocking antibody (FasL Ab) to determine whether FasL was a key causative factor. Compared with isotype control Ab, FasL Ab attenuated renal IRI in both groups. Moreover, FasL blockade eliminated the difference between HIF-2α−/− and WT mice (Figure 5, B–D). Renal samples from HIF-2α−/− mice harvested at 3 hours after reperfusion were also evaluated by immunohistofluorescence staining. As shown in Figure 5E, FasL Ab efficiently inhibited the infiltration of HIF-2α−/− NKT cells into ischemic kidneys.

The Adenosine A2A Receptor (Adora2a) Agonist CGS21680 Lost Protective Effect against Renal IRI in HIF-2α−/− Mice

The protective effect of adora2a activation on IRI25 is based on compromised T/NKT cell activation.6,8 We next subjected HIF-2α−/− mice to an adora2a agonist CGS21680, followed by renal IR. As shown in Figure 6, CGS21680 lost effect in HIF-2α−/− mice, indicating that HIF-2α was necessary for the anti-inflammatory effect of adora2a activation. Consistently, CGS21680 also lost effect in Mx1-HIF-2α−/− mice (data not shown).

NKT Cells, HIF-2α, and Renal IRI
extracts from WT thymocytes. As shown in Figure 8D, only HIF-2α antibody was able to supershift the protein-DNA complex. These observations were consistent with the finding that HIF-2α was constitutively stabilized in the thymus (Supplemental Figure 2), and indicated that HIF-2 played a part in the expression of adora2a in the thymus.

Adora2a Activation-Induced cAMP Production and FasL Inhibition in NKT Cells were HIF-2α-Dependent

Adora2a activation stimulates intracellular cAMP production and functions through it.30 As shown in Figure 9A, CGS21680 induced much more cAMP production in thymocytes than in splenocytes or liver MNCs, which was consistent with the observation that the thymuses had much higher adora2a expression (Figure 8A). The cAMP increase in thymocytes was markedly attenuated by HIF-2α KO. However, the cAMP increase in splenocytes and liver MNCs was abrogated only in Mx1-Cre-mediated HIF-2α−/− cells, probably because the spleen and liver contained immune cells other than T/NKT cells that were not responsive to Lck-Cre-mediated gene disruption and thus could still respond to CGS21680 treatment.

As stated above, HIF-2α KO increased FasL expression on peripheral NKT cells. To determine whether this was a result of compromised adora2a expression/activation, we employed an in vitro model simulating oxidant stress by hydrogen peroxide in a similar fashion to previous reports.31 Because thymic NKT cells from both HIF-2α−/− and WT mice were FasL-negative without treatment (Figures 1 and 9B), thymocytes were used. CGS21680 was added to the culture medium of thymocytes, which were

Figure 4. In vivo NK1.1+ cell depletion eliminated the difference between HIF-2α−/− mice and WT littermates. Mice were injected intraperitoneally with 250 μg anti-NK1.1 monoclonal antibody (PK136) or isotype-matched control IgG2a at 48 hours before ischemic insult (20 minutes). (A) NK1.1+ cell depletion was confirmed by flow cytometry analysis. (B) Serum creatinine levels at 24 hours after the reperfusion (n=6 per group). *P<0.05 versus isogenic mice treated with control IgG. (C) Survival of mice after PK136/control IgG pretreatment and 20 minutes of renal ischemia (n=10 per group). Compared with control IgG, PK136 led to a significant survival advantage in HIF-2α−/− mice by Kaplan-Meier analysis (log-rank test, P<0.05). (D) Representative PAS-stained sections in post-ischemic kidneys harvested at 24 hours. Original magnification, ×200. PAS, periodic acid–Schiff.
then subjected to stimulation by hydrogen peroxide. As shown in Figure 9B, hydrogen peroxide significantly promoted FasL expression on HIF-2α−/− and WT NKT cells. However, CGS21680 attenuated FasL expression on WT but not HIF-2α−/− NKT cells. These results suggested that the up-regulated FasL expression on peripheral HIF-2α−/− NKT cells might be a result of adora2a deficiency and irresponsiveness to endogenous adenosine.

CGS21680/LPS Produced a Remarkable Synergistic Effect, Leading to Greatly Reduced Renal IRI, which was Dependent on HIF-2α

We previously demonstrated that LPS induced endothelial HIF-2α, which protected against ischemic injury.19 In this study, a combination of LPS/CGS21680 reduced the renal injury to a surprising extent in WT mice, but had no effect in Mx1-HIF-2α−/− mice (Figure 10). These results highlight the role of HIF-2α as a key protector in renal IRI, by both preserving endothelial function and reducing inflammation.

DISCUSSION

There is increasing recognition that ischemia/hypoxia may not only impair cellular energy production, but also has a regulatory impact on cellular functions. Central to hypoxia adaptation are HIFs, which have many target genes and generally increase oxygen delivery and/or improve cell survival in conditions of limited oxygen availability. However, the impact of HIF induction on the functions of specific cell types must be determined specifically.

Another mechanism that cells use for adapting to hypoxia is adenosine. It is well known that ischemic tissue damage is accompanied by adenosine accumulation
as a consequence of local hypoxia. Adenosine signals through four receptors, among which adora2a is the major immunoregulatory arm of the adenosine signaling system, and a potent protective molecule in vivo capable of blocking inflammation. Although adora2a is located in the kidney, the protection afforded by adora2a agonists is an effect on T/NKT cells in ischemic renal injury.

So far, several studies have suggested an important link between HIFs and adenosine-related molecules. For example, HIF-1 regulates A2B adenosine receptor expression. HIF-1 also regulates CD73, which converts AMP to adenosine. Previous studies revealed that HIF-2α was associated with adora2a expression in endothelial and chromaffin cells. In this study, we found the adora2a protein level and responsiveness to CGS21680 in immune cells were correlated with HIF-2α.

Our results are important, not only because we reconfi rmed the regulatory relationship between HIF-2α and adora2a in other cell types, but also because these findings provide new evidence and explanations to the well known “hypoxia-adenosinergic immunosuppression”.

The fact that the thymus has a high HIF-2α protein level but no detectable HIF-1α (Supplemental Figure 2) is intriguing because the thymus is an essential organ for the generation, development and maintenance of T/NKT cell immunity. However, this is not surprising given the fact that, compared with other lymphoid organs, the thymus is hypoxic. Previous reports have indicated that HIF-1α primarily governs acute hypoxic responses, whereas HIF-2α functions under physiologic oxygen conditions and at prolonged hypoxia, which explains the difference in the expression patterns between the two HIFs in the thymus. Actually, HIF-1α overexpression leads to cell death in thymocytes.

It was reported that T cell function was highly dependent on their localization and the extent of T cell activation and proliferation was decreased in environment with low oxygen tension. This was in line with our observation that adora2a expression/activation paralleled with hypoxia/HIF-2α in lymphoid organs, and suggested that the hypoxic environment and constitutive HIF-2α stabilization might be partially

Figure 6. The effect of CGS21680 on renal IRI in HIF-2α−/− mice and WT littermates. CGS21680 (0.7 mg/kg) or vehicle (DMSO) was administered intraperitoneally at 24 hours before renal IR. Then the mice were subjected to 20 or 25 minutes of ischemia. The mice were subjected to 20 or 25 minutes of ischemia as described above. (A) Serum creatinine concentrations at 24 hours after reperfusion (n=8 per group). *P<0.05 versus vehicle-treated WT mice. (B) Survival of HIF-2α−/− and WT mice after renal ischemia (n=20 per group). CGS21680 treatment led to a significant survival advantage in WT but not HIF-2α−/− mice by Kaplan-Meier analysis (log-rank test, P<0.05 between CGS21680 and vehicle-treated WT mice in 25 min ischemia group; P>0.05 between CGS21680 and vehicle-treated WT mice in 25 min ischemia group in both 20 and 25 min groups). (C) Representative PAS-stained sections in post-ischemic kidneys harvested at 24 hours. Original magnification, ×200. Abnormalities based on PAS-stained sections were graded and data are expressed as mean±SD from eight mice per group. *P<0.05 versus vehicle-treated WT mice. PAS, periodic acid–Schiff.
responsible for the relatively stable and immune-privileged environment in the thymus. Moreover, given the fact that tissue inflammation/destruction is always accompanied by prolonged local hypoxia, our results also suggest that hypoxia/HIF-2α/adora2a axis may serve as a molecular “brake” system to control undue inflammation through its effect on NKT cells.

It was reported27,28 that adora2a activation in T cells could inhibit their FasL expression and cytotoxicity. However, no reports correlated adora2a activation with FasL inhibition in NKT cells. We found that adora2a expression in thymocytes, splenocytes and NKT cells was HIF-2α-dependent. However, HIF-2α KO-induced FasL over-expression was observed in peripheral NKT cells but not in T cells. Why?

As we know, T/NKT cell activation, and FasL upregulation as a result, is a TCR-triggered event. T cells have highly variant TCRs specific for numerous peptide antigens. Adal isoforms with FasL expression and cytotoxicity. However, no reports correlated adora2a activation with FasL inhibition in NKT cells. We found that adora2a expression in thymocytes, splenocytes and NKT cells was HIF-2α-dependent. However, HIF-2α KO-induced FasL over-expression was observed in peripheral NKT cells but not in T cells. Why?

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mechanisms regulating inflammation in the setting of ischemic renal injury.

CONCISE METHODS

Mice

The Cre/loxP recombination system was used to generate HIF-2α KO mice, as described previously. Lck-Cre (stock number: 003502), Mx1-Cre (003556) transgene mice, HIF-2α floxed mice (008407), as well as Rag1KO mice (002216) were all from The Jackson Laboratory (Bar Harbor, ME). The mating strategy, genotyping and the construction of transgenic mice (002216) were all from The Jackson Laboratory (Bar Harbor, ME). The Cre/loxP recombination system was used to generate HIF-2α KO mice. Mice (002216) were all from The Jackson Laboratory (Bar Harbor, ME). The Cre/loxP recombination system was used to generate HIF-2α KO mice. Mice (002216) were all from The Jackson Laboratory (Bar Harbor, ME). The Cre/loxP recombination system was used to generate HIF-2α KO mice. Mice (002216) were all from The Jackson Laboratory (Bar Harbor, ME).

Male mice, 8–14 weeks of age and weighing 20–28 g, were used in the present study. All animal experiments were conducted according to NIH guide for the care and use of laboratory animals and the institutional guidelines of Shanghai Jiao Tong University School of Medicine. All the procedures described were approved by the Animal Use and Care Committee of Shanghai Jiao Tong University School of Medicine (approval number: SYKX–2012–0013).

Renal Ischemia-Reperfusion (IR) and Drug Treatment

A warm renal IR model was used as described. The selective adora2a agonist, CGS21680 (0.7 mg/kg, dissolved in DMSO; Sigma-Aldrich, St. Louis, MO) was administered intraperitoneally at 24 h before renal IR. In some experiments, CGS21680 was given together with LPS (3 mg/kg, intraperitoneally, from Escherichia coli serotype 055:B5; Sigma-Aldrich). The details of the surgical operation and the application of pharmacologic agents are described in the Supplemental Material.

Whole Body Exposure to Low Oxygen Atmosphere

Some mice were placed in air-tight modular incubation chambers (Shanghai Alcott Biotech Co. Ltd., China) and the atmosphere was controlled by a constant gas flow (1.5 L/min) containing 21% or 10% O2.

Preparation of Single-Cell Suspensions from Lymphoid Organs, Oxidative Stress Treatment and Flow Cytometry Analysis

Single-cell suspensions from spleens and thymuses were prepared by running specific gentleMACS programs on a gentleMACS dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer’s optimized protocols. Liver MNCs were prepared by using the gentleMACS dissociator according to the manufacturer’s protocol and a previous report, with modifications. Isolation of the infiltrating inflammatory cells from the ischemic kidneys was achieved by running specific gentleMACS programs on the gentleMACS dissociator, followed by positive selection using CD45 microbeads (130–052–301; Miltenyi Biotec). The details are described in the Supplemental Material.

The cell suspensions were incubated with anti-mouse CD16/CD32 blocking antibody prior to staining. Then, samples were labeled using the intracellular fixation and permeabilization step by using the intracellular fixation and permeabilization buffer set (88–8824; eBioscience) according to the manufacturer’s protocol. Immunofluorescence staining was analyzed using a FACSCalibur instrument (BD Biosciences, San Jose, CA). The lymphocytes were gated using forward and side scatter to exclude

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Figure 8. Hypoxia-induced adora2a expression in splenocytes, thymocytes, and NKT cells was dependent on HIF-2α. WT and Mx1-HIF-2α+−/− mice were placed in air-tight modular incubation chambers either under normoxic (21% oxygen) or hypoxic conditions (10% oxygen) for 6 hours followed by 6 hours in 21% oxygen. Then spleens and thymuses were harvested. (A) Expression of adora2a was evaluated by western blot analysis and co-detection of β-actin was performed to assess equal loading (n=4 for each group). (B) Adora2a protein bands were quantified and normalized to β-actin. Data are expressed as means±SD. *P<0.05 versus the WT controls. (C) Single-cell suspensions were prepared from spleens. After the fixation and permeabilization step, the expression of adora2a was analyzed on electronically gated NK1.1+TCR-β+ (NKT) cells. Blue lines indicate the staining of adora2a antibody, and red lines indicate the background staining with isotype-matched control IgG. Similar results were obtained in four independent experiments. (D) Thymuses were harvested from normoxia or hypoxia-treated WT mice and nuclear extracts were isolated and subjected to gel mobility shift assays using a probe from mouse adora2a promoter sequence. HIF-1α or HIF-2α antibodies were added to the reaction to generate supershifts. The protein-DNA and supershifted complexes are indicated, respectively.
debris and dead cells, and then 30,000 events were acquired in each assay for analysis.

In some experiments, CGS21680 (10 μmol/L) or DMSO (vehicle) were added to the culture medium of thymocytes at 1 hour before H₂O₂ (25 μmol/L) was added. Four hours later, the cells were labeled with the antibodies (NK1.1, TCR-β and FasL) and subjected to FACS analysis.

**Figure 9.** CGS21680-induced cAMP production and FasL inhibition in HIF-2α−/− and WT immune cells. (A) WT, HIF-2α−/− and Mx1-HIF-2α−/− mice received no previous treatments before the splenocytes, liver MNCs and thymocytes were prepared and treated with CGS21680 (10 μmol/L) or vehicle (DMSO). Intracellular cAMP level was determined by ELISA. *P<0.05 versus vehicle-treated controls. **P<0.05 versus CGS21680-treated WT thymocytes. (B) CGS21680 or DMSO was added to thymocyte suspensions at 1 hour before H₂O₂ (25 μmol/L) or PBS was added. Four hours later, the expression of FasL was analyzed on electronically gated NK1.1 TCRIβ (NKT) cells. Blue lines indicate the staining of FasL antibody, and red lines indicate the background staining with isotype-matched control IgG. Similar results were obtained in four independent experiments.
Each section was observed under a confocal laser scanning microscope at a magnification of ×200.

**Measurement of Intracellular cAMP Accumulation**

Intracellular cAMP level was detected by using a Monoclonal Antibody-Based Direct cAMP ELISA Kit (NewEast Biosciences, Malvern, PA), as described previously. The details are described in the Supplemental Material.

**Measurement of Creatinine and BUN**

Serum creatinine (Cr) and BUN levels were measured via a standard clinical automatic analyzer (Siemens Dimension Xpand; Dade Behring, Marburg Germany).

**Histomorphological Analyses**

Paraffin-embedded kidney tissues were stained with periodic acid–Schiff, processed for immunohistochemical localization of myeloperoxidase, or subjected to TUNEL assay. Details are in the Supplemental Material.

**Western Blot Analyses**

Western blot analyses of HIF-1α (NB100–134; Novus Biologicals), HIF-2α (ab199; Abcam, Inc.), adora2a (sc-13937; Santa Cruz Biotechnology) and FasL (ab15285; Abcam, Inc.) were performed as described previously. Detailed procedures are described in the Supplemental Material.

**EMSA**

Protein-DNA interaction was detected by using an Odyssey Infrared EMSA Kit (LI-COR, Lincoln, NE), according to the manufacturer’s protocols. Briefly, nuclear extracts from thyrmuses were assembled with a DyLight 680-labeled double-stranded DNA probe (Takara Co., Dalian, China) from mouse adora2a promoter sequence, which was located about 34 bp upstream of mouse adora2a exon 1, as described in a previous report. The sense sequence was 5’-GGACGGGTGGACCTGAGCCGCCCAGTTGGGG-3’. The signal was detected and quantified with Odyssey infrared imaging system (LI-COR). Supershift assays using HIF-1α or HIF-2α antibody were also conducted to confirm the specificity of HIF/DNA-binding activity. The details are described in the Supplemental Material.
Statistical Analyses
All values were reported as means±SD. Data were analyzed by one-way ANOVA with a subsequent Student–Newman–Keul’s test, or a t test where applicable. Statistical significance was set at P<0.05.

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Hypoxia-Inducible Factor-2α Limits NKT Cell Cytotoxicity in Renal Ischemia/Reperfusion Injury

Supplementary Results

There Was a Constitutive HIF-2α Stabilization in the Thymus, Which Can Be Abrogated by Lck-Cre-mediated HIF-2α Knockout

Genomic PCR analysis indicated efficient recombination of the HIF-2α conditional allele in NKT cells (Supplementary Figure 1). Western blot analysis showed that without any treatment wild-type (WT) thymus had a relatively high expression of HIF-2α, which couldn’t be detected in the mutants (Supplementary Figure 2). Measurements of renal function parameters and erythropoiesis revealed no significant difference between HIF-2α−/− mutants and WT littermates without treatment (Supplementary Table 1).

Complete methods

Mice

The Cre/loxP recombination system was used to generate HIF-2α knockout mice, as described previously.1 Lck-Cre (stock number: 003802), Mx1-Cre (003556) transgene mice, as well as HIF-2α floxed mice (008407) were all from the Jackson Lab (Bar Harbor, Maine USA). After a mating of HIF-2αloxP/loxP with these Cre transgene strains and a second mating of their progeny, mice that were homozygous for the HIF-2α floxed allele and also carried the Cre transgene were generated. Lck-Cre+HIF-2αloxP/loxP mice were referred to as HIF-2α−/− mutants with mutated HIF-2α in T-lineage cells, including NKT cells. Mx1-Cre+HIF-2αloxP/loxP mice that had received 3 injections of an interferon inducer poly deoxyinosinic/deoxycytidylic acid (pIpC, 400μg) were referred to as Mx1-HIF-2α−/− mice. Cre-negative littermates were used as WT controls. Rag1KO mice (002216) were also from the Jackson Lab (Bar Harbor, Maine USA)

Genotyping and the confirmation of target gene excision were as described previously.2,3 Briefly, the WT, 2-loxP, and 1-loxP allele (KO allele) were distinguished by a multiplex PCR (P1: 5’-CAGGCAGTATGCCTGGCTAATTCCAGTT-3’; P2: 5’-CTTCCTTCCATCATCTGGGATCTGGGACT-3’; P3: 5’-GCTAACACTGTACTGTCTGAAAGAGTAGC-3’). The WT allele produced a 410-bp fragment (P1 and P2), the 2-loxP allele produced a 444-bp fragment (P1 and P2), and the 1-loxP allele produced a 340-bp fragment (P1 and P3). To confirm the successful HIF-2α knockout in different tissues by Cre recombinase, thymus, liver, spleen, lymph node samples, as well as purified NKT cells from Mx1-HIF-2α−/−, Lck-HIF-2α−/− and Cre+HIF-2αloxP/loxP mice were subjected to PCR analysis, and a typical result was shown in Supplementary Figure 1.

Male mice, 8-14 weeks of age and weighing 20-28g, were used in the present study. All animal experiments have been conducted according to NIH guide for the care and use of laboratory animals and the institutional guidelines of Shanghai Jiaotong University.
School of Medicine. All the procedures described were approved by the Animal Use and Care Committee of Shanghai Jiaotong University School of Medicine (approval number: SYKX-2012-0013). All surgery was performed under sodium pentobarbital anesthesia. Analgesia used was bupivacaine (0.5%), a long acting local analgesic, immediately after surgery and only once. Several drops of bupivacaine were dripped on the suture line after the muscle layer was closed, and before the closure of skin wound. All these efforts were made to minimize suffering.

Renal Ischemia-Reperfusion (IR) and Drug Treatment

The selective adora2a agonist, CGS21680 (0.7mg/kg, from Sigma-aldrich), was administered i.p. at 24 h before renal IR. In some experiments, CGS21680 was given together with lipopolysaccharide (LPS, 3mg/kg, i.p., from E. coli serotype 055:B5, Sigma-aldrich).

A warm renal IR model was used as described previously,\(^1\), \(^2\) with minor modifications. The surgical procedures were carried out by an experienced investigator with no prior information regarding the previous treatments and genetic background of the animals. Animals were anesthetized with sodium pentobarbital (60 mg/kg body weight i.p.) and placed on a temperature-controlled heating table with a rectal thermometer probe attached to a thermal feedback controller (ALC-HTP Homeothermic System, Shanghai Alcott Biotech Co. Ltd, China) to maintain rectal temperature at 36°C. Following a midline abdominal incision, right nephrectomy was performed. After intraperitoneal injection of heparin (50 U/kg), left renal pedicle was localized and clamped for 20 or 25 min using an atraumatic micro-vascular clamp. After inspection for signs of ischemia, animals were covered with surgical dressing to keep stable intraperitoneal temperature. After removal of the clamp, restoration of blood flow was inspected visually. Mice underwent same surgical procedures but without vascular occlusion, hereafter were referred to as sham controls. Animals were killed 3 h, 6 h, 9 h, 12 h or 24 h after reperfusion by exsanguination, to obtain blood and renal samples for further analyses.

Separate groups of mice were used in the survival experiments and survival was recorded daily. If an animal was considered possibly morbid during the observation period, the condition of the animal was monitored every two hours. The presence of morbid symptoms was determined by an experienced observer with no prior information regarding the treatments and genetic background of the animals. Animals were considered morbid if they were severely immobile, hunched in posture, experiencing severe hypothermia, and/or unresponsive to noise. After signs of morbidity were detected, death was considered unavoidable and the animal was euthanized via exsanguinations under anesthesia. Renal failure was confirmed by macroscopic and microscopic examination. The animals that survived to 7 days after reperfusion were euthanized via exsanguinations under anesthesia, and the successful recovery of renal function was confirmed by serum and histological analyses.

Preparation of Single-Cell Suspensions from Lymphoid Organs, Oxidative Stress Treatment and Flow Cytometry Analysis
After mice were euthanized under anesthesia, spleens, thymuses and livers were removed. Splenocytes and thymocytes were prepared by running specific gentleMACS programs on a gentleMACS dissociator (Miltenyi Biotec Inc, Bergisch Gladbach, Germany), according to the manufacturer’s optimized protocols. Liver MNCs were prepared by using the gentleMACS dissociator according to the manufacturer’s protocol and a previous report, with modifications. Briefly, the liver was perfused in situ through the portal vein with room temperature PBS. Then the liver was cut into small pieces in prewarmed dissociation mix solution (Krebs-Ringer-Buffer solution supplemented with CaCl₂, MgCl₂, Collagenase IV and DNase I). The liver was transferred into the C Tube, which was attached upside-down onto the sleeve of the gentleMACS dissociator and run the gentleMACS program m_liver_01.02. The sample was incubated for 30 min at 37°C under slow continuous rotation using the MACSmix Tube Rotator. Then run the gentleMACS Program m_liver_02.02. The liver sample was pressed gently through a 70μm cell strainer (BD Falcon, #352350), and suspended in 40 ml of cold PBS/FBS/Az solution. The pellet was then resuspended in a 37.5% isotonic Percoll solution and centrifuged at 680–700g for 12 min at room temperature. The cells of interest would form a pellet at the bottom of the solution. The supernatant was discarded and the pellet containing RBC and lymphocytes was washed in cold PBS/FBS/Az solution. After the RBCs were lysed, the cells were resuspended in 5 ml PBS/FBS/Az and filtered through 70μm cell strainer again to remove the debris. Finally, the mononuclear cells (MNCs) were resuspended in PBS/FBS/Az solution, tissue culture medium, or staining buffer, according to the downstream experiment. When purified from an untreated mouse, an average of 4–5×10⁶ MNCs (60–70% of them being lymphocytes) can be recovered with this method.

The single-cell suspensions were incubated with anti-mouse CD16/CD32 blocking antibody prior to staining. Then samples were labeled using combinations of the following antibodies: anti-NK1.1 APC (17-5941, eBioscience), anti-TCR-β FITC (11-5961, eBioscience), anti-FasL PE(12-5911, eBioscience) or anti-adora2a PE (sc-32261, Santa Cruz). Because the adora2a antibody was against the adora2a protein epitope mapping to the intracellular loop, it was added to the samples after the fixation and permeabilization step by using the intracellular fixation and permeabilization buffer set (88-8824, eBioscience) according to the manufacturer’s protocol. Immunofluorescence staining was analyzed using a FACSCalibur instrument (BD Biosciences). The lymphocytes were gated using forward and side scatter to exclude debris and dead cells, then 30,000 events were acquired in each assay for analysis.

In some experiments, CGS21680 (10μmol/L) or DMSO (as vehicle) were added to the culture medium of suspended thymocytes at 1h before H₂O₂ (25μmol/L) was added. 4 hours later, the cells were labeled with the antibodies (NK1.1, TCR-β and FasL) and subjected to FACS analysis.

**Isolation of the Infiltrating Inflammatory Cells from the Ischemic Kidneys**

Inflammatory Cells from the ischemic mouse kidneys were isolated by using the gentleMACS Dissociator (Miltenyi Biotec), according to the protocol described by the manufacturer. In brief, HIF-2α−/− mice and their WT littermates were subjected to
bilateral renal ischemia (20 min), followed by 3 h of reperfusion. Then both kidneys were harvested and capsule was removed. After the kidneys were transferred into the digest solution in the gentleMACS C Tube, which was attached to the gentleMACS Dissociator, we ran certain programs (m_lung_01 and m_spleen_04) according to the instructions, to dissociate the kidneys. Then a cell strainer (70 µm mesh size) was applied to remove the tissue debris. The obtained cells were counted and magnetically labeled with CD45 microbeads (Miltenyi Biotec, 130-052-301). The unwanted cells were subsequently depleted by separation over a MACS Column, which was placed in the magnetic field of the MACS Separator. After removal of the column from the magnetic field, the magnetically retained CD45+ cells were eluted and subjected to FACS analysis as described above.

NKT Cell Purification from Splenocytes and Adoptive Transfer

NKT Cell isolation was performed in a two-step procedure from mouse spleens using a commercially available NK1.1+ iNKT Cell Isolation Kit (130-096-513, Miltenyi Biotec Inc, Bergisch Gladbach, Germany), according to the manufacturer’s protocols. Briefly, HIF-2α−/− mice and their WT littermates were euthanized under anesthesia and spleens were harvested. Splenocytes were passed through a 40-mm nylon cell strainer (BD Biosciences) and collected in phosphate buffered saline. Red blood cells were lysed, and the non-NK1.1+ iNKT cells are labeled with a cocktail of biotin-conjugated antibodies, anti-Biotin MicroBeads. The labeled unwanted cells, including NK cells, B cells, macrophages, CD8+, and TCRγδ+ T cells were subsequently depleted by separation over a MACS Column, which was placed in the magnetic field of the MACS Separator. Then the pre-enriched NK1.1+ iNKT cells were labeled with anti-NK1.1-APC and anti-APC microbeads and then positively selected by magnetic separation to produce cell populations of ≥90% NK1.1+ iNKT Cells (Supplementary Figure 3). About 3×10^5 NKT cells (from 4-6 mice) were adoptively transferred into a Rag1KO mouse via tail vein injection at 4 d before renal IRI. Successful reconstitution was confirmed by FACS analysis of splenocytes collected after 24 h of reperfusion. Control animals received vehicle injections which didn’t contain cells.

Measurement of Intracellular Cyclic AMP (cAMP) Accumulation

Splenocytes, liver MNCs or thymocytes were treated with 50µM 3-isobutyl-1-methylxanthine (IBMX, Sigma-Aldrich, USA) for 30 min at 4°C, followed by culture in 37°C incubator. Then CGS21680 (10µmol/L) or the equivalent amount of vehicle (DMSO) was added to the culture medium. At 60 min after the treatment, the culture medium was removed and 1N HCl was added to stop endogenous phosphodiesterase activity and achieve adequate cell lysis. Then the mixture were boiled for 5 minutes and centrifuged at 10000 rpm for 5 minutes at 4°C. The supernatant was collected and the cAMP level was determined by using a Monoclonal Anti-cAMP Antibody-Based Direct cAMP ELISA Kit (NewEast Biosciences Inc., Malvern, USA).

Histology and Histomorphological Scoring of Acute Tubular Injury
Kidney tissues were fixed in 10% neutral buffered formalin overnight, dehydrated, embedded in paraffin and sectioned at 3 μm. For histological analysis, sections were stained with Periodic Acid-Schiff (PAS). Samples were analyzed for tubular cell necrosis, tubular dilation, intratubular cell detachment, and cast formation (original magnification ×200) and were all evaluated in a blinded manner by a nephropathologist. Abnormalities were graded by a semiquantitative histomorphological scoring system from 0 to 4, as described previously.1,2 At least 3 fields per section were evaluated.

**Polymorphonuclear Leukocyte Infiltration (MPO Activity)**
Renal sections were processed for immunohistochemical localization of myeloperoxidase (MPO, polyclonal rabbit antibody; Novus Biologicals, NBP1-42591), and were then visualized with diaminobenzidine (DAB) and counterstained with hematoxylin. Polymorphonuclear leukocyte (PMN) infiltration was scored semiquantitatively on a scale of 1 (none) to 4 (severe), as described previously.1,2

**Terminal Deoxynucleotidyl Transferase-Mediated 2'-Deoxyuridine 5'-Triphosphate Nick-End Labeling Assay (TUNEL)**
Apoptotic cells in formalin-fixed, paraffin-embedded kidney tissue sections were identified with ApopTag Fluorescein In Situ Apoptosis Detection Kit (S7110, Chemicon International), according to the manufacturer’s protocol. Cells with nuclear positive staining by fluorescent antibodies for DNA fragmentation were visualized directly by a fluorescence microscopy and counted (original magnification ×200). At least 3 fields per section were examined.

**Western Blot Analyses**
Western blot analysis of HIF-1α/HIF-2α was performed as described previously.1,2 Nuclear extracts were isolated from harvested thymuses and spleens using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Inc., USA), supplemented with Complete Protease Inhibitor Cocktail Tablets (Roche, Indiana, USA). Protein samples were separated on 10% SDS-PAGE gels and then transferred to a nitrocellulose membrane (Whatman) by standard procedures. The membranes were then incubated with rabbit polyclonal primary antibodies against HIF-1α (1:500, NB100-134, Novus Biologicals), HIF-2α (1:500, ab199, Abcam) or a loading control TATA binding protein (TBP, 1:2000, ab818, Abcam), and then with secondary antibodies (1:10000; LI-COR Biosciences). Signals were visualized and detected using an Odyssey infrared imaging system (LI-COR Biosciences). Samples were corrected for background and quantified using Odyssey software. All values were normalized to the loading control and expressed as fold increase relative to control.

For adora2a and FasL measurements, harvested kidneys, spleens and thymuses were homogenized and lysed with cell lysis buffer, which contained 1 protease inhibitor cocktail tablet per 10 mL of Lysis Reagents (Complete; Roche, Indianapolis, IN). Solutions were then clarified by centrifugation (25 min at 16,000g). Solubilized proteins were then resolved on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose membranes (Whatman). After blocked with LI-COR blocking buffer, blots were
incubated with adora2a (1:500, sc-13937, Santa Cruz), FasL (1:200, ab15285, Abcam) and anti-β-actin (1:2000, Santa cruz) antibodies. After incubation with secondary antibodies, blots were developed as described above.

**Electrophoretic Mobility Shift Assay (EMSA)**

Protein-DNA interaction was detected by using an Odyssey Infrared EMSA Kit (LI-COR, Lincoln, NE), according to the manufacturer's protocols. Briefly, nuclear extracts were isolated from harvested thymus using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Product Number 78833, Pierce Biotechnology, Inc., USA.), supplemented with Complete Protease Inhibitor Cocktail Tablets (Roche, Indiana, USA). For EMSA, 10μg of nuclear protein were assembled with 5×Gel Shift Binding buffer (20% glycerol, 5 mmol/L MgCl₂, 2.5 mmol/L EDTA, 2.5 mmol/L DTT, 250 mmol/L NaCl, 50 mmol/L Tris-HCl), 0.25 mg/ml poly(dI)-poly(dC), and 5’ DyLight 680-labeled oligonucleotide from mouse adora2a HRE (Takara, Dalian, China), which was located in the promoter sequence of mouse adora2a (about 34 bp upstream of exon 1). The sense sequence was GGACGCGTGGACCTGAAGCGCCCACGTTGGGG. Both sense and antisense DNA oligonucleotides were labeled with DyLight 680 at 5’ end and annealed to form a double-stranded DNA fragment by placing the oligonucleotide set in a 100°C heat block for 5 minutes. After incubation at room temperature for 30 minutes, the samples were loaded on a pre-run 8% polyacrylamide gel and electrophoresis was continued at 30 mA for 90 min. The signal was then detected and quantified with Odyssey infrared imaging system (LI-COR). Supershift assays using HIF-1α or HIF-2α antibodies were also conducted to confirm the specificity of HIF/DNA-binding activity. For loading control, 10μg of nuclear proteins from each sample were subjected to western blot analysis, which showed equal loading.

**REFERENCES**

Supplementary Table 1. Analysis of physiologic parameters in 10-week-old male HIF-2α−/− mice and the Cre− (WT) littermates.

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<th>Parameter</th>
<th>Cre−</th>
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<th>Mx1-HIF-2α−/−</th>
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<td>Body weight (g)</td>
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<td>Kidney weight (mg)</td>
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<td>Hgb (g/L)</td>
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<td>PLT (10^9/L)</td>
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<td>BUN (mmol/L)</td>
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<td>Cr (μmol/L)</td>
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<td>31±6.7</td>
<td>35±6.7</td>
<td>&gt;0.05</td>
</tr>
</tbody>
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Values represent means and SD of 4 mice of each group.
Supplementary Figure 1. Confirmation of HIF-2α knockout by polymerase chain reaction (PCR). Thymus, liver, spleen, lymph node samples, as well as purified NKT cells from Mx1-Cre+HIF-2α<sup>loxP/loxP</sup>, Lck-Cre+HIF-2α<sup>loxP/loxP</sup> and Cre-HIF-2α<sup>loxP/loxP</sup> mice were subjected to a multiplex PCR, which produced a 410-bp fragment in WT allele, a 444-bp fragment in the 2-loxP allele and a 340-bp fragment in the 1-loxP allele (Δ). DNA from the tail of a wild-type mouse was used to show where wild-type HIF-2α was (WT, 410bp).
Supplementary Figure 2. (A) HIF-1α/HIF-2α expression in the thymuses and spleens of HIF-2α−/− mice and WT littermates. The mice received no previous treatments before the thymuses and spleens were harvested. Nuclear HIF-1α/HIF-2α expressions were evaluated by western blot analysis and co-detection of TBP was performed to assess equal loading (n = 4 for each group). No stabilization of HIF-1α was observed in the thymuses or spleens. HIF-2α protein bands were quantified and normalized to TBP. Data were expressed as means ± SD. *, P < 0.05 versus WT thymuses.
Supplementary Figure 3. Purification of NKT cells from splenocytes. After the single-cell suspensions from spleens were prepared and before the purification procedures, the cells were subjected to FACS analysis and a typical result was shown on the left. Then NKT cell isolation was performed in a two-step procedure from splenocytes using a commercially available NK1.1+ iNKT Cell Isolation Kit. A typical post-isolation result was shown on the right. Typically this method produced cell populations of >90% NKT cells from splenocytes.