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Hypoxia stimulus: An adaptive immune response during dendritic cell maturation

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The 'injury hypothesis' in organ transplantation suggests that ischemia–reperfusion injury is involved in the adaptative alloimmune response. We previously found that a strong immune/inflammatory response was induced by ischemia during kidney transplantation in rats. We show here that immature dendritic cells (DCs) undergo hypoxia-mediated differentiation comparable to allogeneic stimulation. Hypoxia-differentiated DCs overexpress hypoxia inducible factor-1α (HIF-1α) and its downstream target genes, such as vascular endothelial growth factor or glucose transporter-1. Rapamycin attenuated DC differentiation, HIF-1α expression, and its target gene expression in a dose-dependent manner along with downregulated interleukin-10 secretion. Coculture of hypoxia-differentiated DCs with CD3 lymphocytes induced proliferation of lymphocytes, a process also neutralized by rapamycin. Furthermore, *in vivo* examination of ischemia–reperfusion-injured mouse kidneys showed a clear maturation of resident DCs that was blunted by rapamycin pretreatment. Our results suggest that hypoxia is a central part of the 'injury hypothesis' triggering DC differentiation under hypoxic conditions. Rapamycin attenuates the hypoxic immune-inflammatory response through inhibition of the HIF-1α pathway.

Kidney International (2008) **73,** 816–825; doi:10.1038/sj.ki.5002792; published online 23 January 2008

KEYWORDS: dendritic cells; hypoxia; mTOR inhibitors; costimulatory molecules; cytokines

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Received 22 January 2007; revised 31 October 2007; accepted 13 November 2007; published online 23 January 2008

During recent years, dendritic cells (DCs) attracted much interest in the medical community due to their strategic role in immune response linking innate and acquired immune responses and in the induction of tolerance to self antigens. In the transplant setting, immune-mediated injury is not only caused by alloimmune response, but also, as referred by the 'injury hypothesis,' as a result of other factors that may play an important role (for example, ischemia–reperfusion injury (IRI)).[1–3] IRI has been shown to activate innate immunity in rat kidneys[4] and clinical data have invariably confirmed that there is a link between delayed graft function and a higher rate of acute rejection.[5] In an experimental transplant model, our group found that ischemia added to the allogeneic background resulted in significant inflammatory injury, clearly activating and accelerating the cellular mechanisms involved in this process.[3] Several reports even corroborate that the immune response vis-à-vis hypoxia can occur in native kidneys, implicating T cells as the main effectors.[6] Moreover, a relationship between upregulation of DC differentiation and ischemic acute renal failure has recently been described.[7] In the reperfusion phase in rats (peripheral blood monocytes) suffering from ischemic acute renal failure, there was an increase in the number of DCs differentiated from PBMo, and higher expression of MHC class II, IFN-γ, and interleukin (IL)-12 in DCs.[7] Thus, there is a growing evidence of the pivotal role of the different steps of immune system in the pathophysiology of IRI, not only concerning T cells.

In association to this, many immunosuppressive agents have been studied so as to interfere with differentiation toward mature DCs. Rapamycin, a macrolide drug used in clinical transplantation by its antiproliferative effects on T lymphocytes, has been shown to reduce antigen uptake by immature DCs (iDCs) and to decrease expression of costimulatory molecules on mature DCs in a dose-dependent way.[6] Rapamycin is also known to reduce proliferative responses to hypoxia in many cell lines.[8,9] Our group previously demonstrated in a rat kidney transplant model that rapamycin regimens prevent immunogenicity derived from early postischemic inflammatory responses.[2]

Thus, in this study, we had two objectives: first, to assess whether *in vitro* exposure to hypoxia triggers iDCs to

undergo differentiation and if rapamycin can mitigate this cell process, thus attenuating the activation of immune response; and second, to evaluate IRI effect in resident DC (rDC) maturation in an *in vivo* model of rodents treated or not with rapamycin.

RESULTS

Phenotype switch of DCs following lymphocyte or hypoxia exposure

In this study, we made use of $CD11c^+$-$CD14^+$ human monocytes, exposing them to granulocyte–macrophage colony-stimulating factor (GM-CSF) and IL-4 (with or without rapamycin), and then subsequently cocultured with lymphocytes on day 6, when their phenotype was compatible with iDCs ($CD14^-$) (data not shown). DC differentiation was monitored at day 7 by flow cytometry using anticostimulatory molecule (anti-CD40/anti-CD80/CD86), myeloid DC molecule (anti-CD11c), and mature phenotype molecule (anti-CD54/anti-CD58/anti-HLADR) staining. A low population of $CD14^-$ cells, and $CD11c^+$ not previously exposed to any other agonist, became CD40 positive at day 7, which indicates that DCs remain immature under control conditions (Figure 1a). By contrast, iDCs exposed to allogeneic or isogeneic lymphocytes showed high expression of CD40 and the other surface molecules (Figure 1b), which is consistent with DC differentiation. We then examined the forward/side scatter, quantified by mature DC phenotype surface molecule staining, which was characterized by a higher cell surface than immature forms. Allogeneic stimulation revealed higher numbers of mature cells (57.3%) compared to stimulation with isogeneic lymphocytes, resulting in a differentiation rate of 37.1%, while nonstimulated cells remained poorly differentiated (6.3%) (mean values for three experiments) ($P<0.05$). DC differentiation by isogeneic stimulation is a characteristic feature of DCs but not macrophages.

Immature DCs exposed for 48 h to hypoxia (0.5% oxygen) at day 5 showed clear CD40 and all the other surface markers (Figure 2) overexpression at day 7 ($P<0.05$). Maturation changes were also observed in the forward/side scatter analysis, amounting to 54.8% (mean value for six experiments) of mature DCs. Time kinetics performed for 12, 24, 48, and 72 h revealed the most prominent changes in iDC differentiation occurring at 48 h, with no differences over prolonged incubation periods (data not shown). The combination of allogenic DC stimulation performed under hypoxia showed no additive response with regard to the phenotype switch.

To further analyze DC differentiation under hypoxia stimulus, we evaluated succinimidyl ester of carboxyfluorescein diacetate (CFSE) staining. The increase in gate R1 of mature forms ($CD40^+/CD11c^+/CD56^+$) under hypoxia was associated to a similar amount of DNA synthesis than the low population of $CD40^+/CD11c^+/CD56^+$ DCs in gate R1 of cells not exposed to stimuli (control conditions) (data not shown). So, hypoxia does not contribute to an increase in $CD40^+/CD11c^+/CD56^+$ DC proliferation, but induces iDC differentiation.

Rapamycin decreased DC differentiation expression in response to hypoxia

Rapamycin was used in further experiments to evaluate mTOR involvement on DC differentiation under hypoxia. This macrolide was added in a dose-dependent manner, with hypoxia-induced differentiation followed by forward/side scatter analysis and quantification by CD40 and the other maturation markers expression. Rapamycin (at a dose of 0.1 nM) significantly reduced DC maturation to 33.2% (mean), while 10 nM rapamycin reduced differentiation to control values (Figures 3 and 4).

At the same time, we monitored $DiOC_6$ and Annexin-V staining to exclude any interference by apoptosis, thereby accounting for the inhibitory effect of rapamycin. Results showed that there was no increase in apoptosis either in $CD14^+$ cells or in mature DCs exposed to rapamycin (data not shown).

DC cytokine secretion under hypoxia and allogenicity: effect of rapamycin

Interleukin-2, -4, -6, -10, TNF-α, and IFN-γ protein secretion was quantitatively measured following cell stimulation by the cytometric bead array (CBA) system. Under hypoxia, only the levels of IL-10 and IFN-γ significantly got increased (Figure 5). Interestingly, the increase in IL-10 secretion was 40% higher than the increase in IFN-γ production (ratio of coefficient of variation of cytokine increase 0.45/0.32). iDCs under hypoxia and exposed to rapamycin showed a significant reduction in IL-10 secretion and a nonsignificant increase in IFN-γ. In contrast, IFN-γ was oversecreted under allogeneic exposure and IL-4 under isogeneic exposure, while rapamycin produced an increase of IL-6 when added to allogeneic coculture.

HIF-1α pathway alterations in hypoxia-differentiated DCs

In the following experiments, it was our intention to monitor expression of vascular endothelial growth factor (VEGF) and glucose transporter-1 (Glut-1) by quantitative reverse transcription-PCR in association with the differentiation induced by hypoxia. VEGF and Glut-1 are considered classical hypoxia inducible factor-1α (HIF-1α) target genes and therefore are indicative of hypoxic responses. As shown in Figure 6a, both VEGF and Glut-1 expression are hypoxia inducible. Glut-1 expression was induced 30-fold, whereas VEGF expression increased roughly 15-fold compared with normoxic controls.

Taking into account that rapamycin attenuated hypoxia-DC differentiation, we also determined the impact of rapamycin on VEGF and Glut-1 expression. In the presence of 5 nM rapamycin, hypoxia no longer generated a significant increase in mRNA, either of VEGF or of Glut-1 (Figure 6a). Thus, our results suggest the presence of an mTOR-dependent pathway facilitating hypoxia-induced gene expression.

Further mRNA expression analysis revealed a threefold increase of HIF-1α mRNA under hypoxia treatment, a

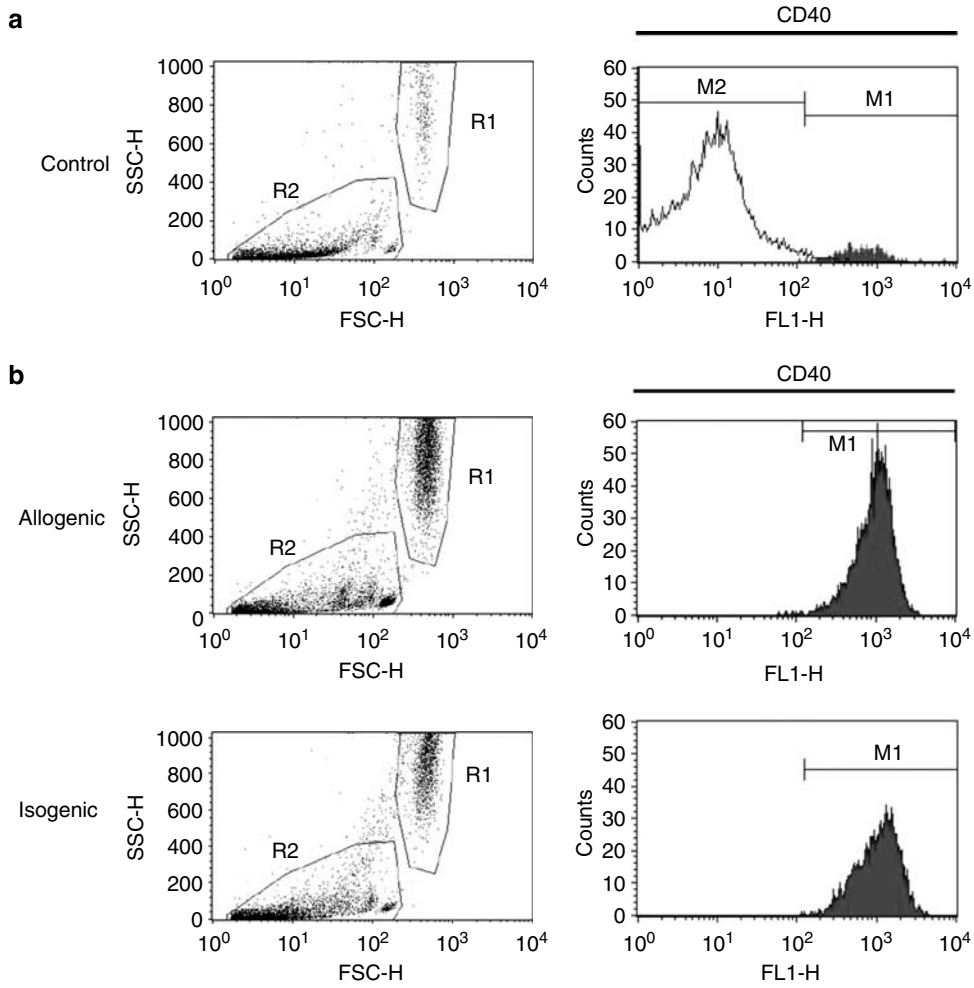

Figure 1 | iDC maturation and CD40 expression under control, allogeneic and isogeneic conditions. (**a**) DC maturation and CD40 expression in control iDCs. Monocytes were cultured with IL-4 and GM-CSF for 5 days. On day 7, cells were harvested and CD11c$^+$ cells were analyzed for CD40 surface marker by flow cytometry. Mean fluorescence intensity of iDCs (not exposed to stimuli for maturation and used as controls) and morphologic changes (FSC-H and SSC-H) were markedly low. The results are representative of three experiments. Gate R1 represents the percentage of mature forms CD11c+; gate R2 cells did not express CD40 and exhibited lower shape and density (immature forms CD11c+); M1 represents positive fluorescence intensity for CD40 staining. (**b**) Allogeneic and isogeneic conditions cause phenotypic and morphologic changes in iDCs. Monocytes were cultured with IL-4 and GM-CSF. On day 6, iDCs were cocultured with lymphocytes from other donors. On day 7, cells were harvested and CD11c$^+$ cells analyzed for CD40 surface marker by flow cytometry. Mean fluorescence intensities of DCs under allogeneic conditions and morphologic changes (FSC-H and SSC-H) were markedly higher than under control conditions. The results are representative of three experiments. Isogeneic stimuli also caused phenotypic changes in iDCs.

situation resembling DC differentiation (Figure 6b). Rapamycin at a dose of 5 nM did not interfere and revealed only insignificant changes on its own. Contrarily to mRNA, HIF-1α protein is more stable under hypoxia, and its quantification is more accurate in this situation as the protein is regulated in a posttranscriptional level. Thus, we monitored protein expression of HIF-1α by western blot analysis (Figure 7). Under hypoxic conditions, in which we observed DC differentiation, we also noted the robust expression of HIF-1α, compared with its complete absence under normoxia. Interestingly, rapamycin (5 nM) attenuated hypoxia-evoked HIF-1α expression. At a dose of 10 nM, rapamycin nearly abrogated hypoxia-evoked HIF-1α expression (data not shown). Thus, blocking the mTOR pathway affects not only DC differentiation but also HIF-1α expression.

To further assess HIF-1α involvement in DC maturation under hypoxia, we exposed iDCs to desferrioxamine, a chemical inducer of HIF-1α stabilization under normoxia. Cell supplementation of DFX 100 μM for 3 h on day 5 was associated with DC differentiation, similarly as hypoxia did (data not shown).

Functional DC evaluation: CD3 lymphocyte proliferation study

To evaluate the effect of DCs differentiated under different conditions on CD3 lymphocyte proliferation, we performed CFSE/CD3 costaining. Lymphocytes were CFSE stained and exposed to mature DCs (under hypoxia or allogeneic stimuli) or exposed to rapamycin-treated hypoxia-DC for 24 h. After 10 days, we analyzed by fluorescence-activated cell sorting

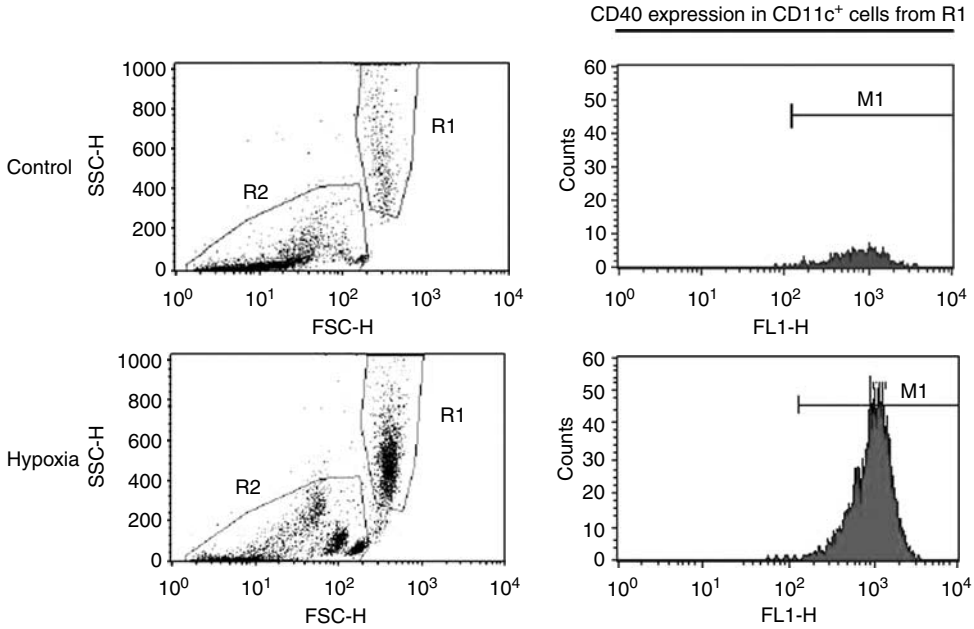

Figure 2 | Hypoxic conditions cause phenotypic and morphologic changes in iDCs. CD40 staining. Monocytes were cultured with IL-4 and GM-CSF. On day 5, iDCs were exposed to 48 h of hypoxia (0.5%). Mean fluorescence intensities of DCs and morphologic changes (FSC-H and SSC-H) were markedly higher than under control conditions and comparable to allogeneic exposure. The results are representative of six experiments.

(FACS) the CFSE staining of CD3 cells that were not exposed to DCs (control) and of CD3 cells exposed to DCs. CD3 lymphocyte proliferation was significantly higher after coculture with hypoxia- or allogeneic-differentiated DCs. Interestingly, proliferation rate was significantly reduced when CD3 cells were cocultured with rapamycin-treated hypoxia-DCs, although those lymphocytes were not directly treated with rapamycin (Figure 8).

Resident intrarenal DCs undergo maturation following ischemia–reperfusion injury

To further evaluate hypoxia-DC differentiation, we verified whether *in vivo* kidney rDCs differentiate after IRI. To distinguish rDCs from other intrarenal cell populations, flow cytometric analysis was performed. Cell suspensions prepared from the kidney 24 h after ischemia, control kidneys, and ischemic kidneys from rapamycin-treated mice (8 mg kg^{-1} for 3 days prior to IRI) were stained for the pan-leukocyte marker CD45 in combination with CD11c, CD80, and either Ly6C and 7-AAD (to distinguish from recruited monocytes).[10] Results of the overall analysis of cell subsets expressed as percentage of the total CD45 are presented in Figure 9. The proportion of viable CD80-differentiated DCs (7-AAD/CD45$^+$/CD11c$^+$/Ly6C$^-$) remained low in control kidneys. There was an increase of viable mature rDCs in ischemic kidneys 24 h after renal artery clipping. Ischemia–reperfusion (IR) was associated with a rich population of CD80 with larger cell size (reflected by forward scatter; data not shown). Thus, rDCs within ischemic kidneys exhibited a more mature phenotype than nonischemic kidneys. Furthermore, rapamycin-ischemic kidneys showed a reduction of viable mature CD80 rDC forms, close to control kidneys.

DISCUSSION

Ischemia–reperfusion injury in solid organ transplantation has been shown to contribute to the adaptive immune response involved in acute rejection (1, 4, and 11). It is well-known that T cells can be activated by oxygen-free radicals and cytokines, both associated with ischemia and, thus, orchestrate the immune inflammatory reaction.[4] Using a Fischer-to-Lewis model of kidney transplant, we recently assessed the distinct contributions of alloreactivity and cold ischemia to the onset and progression of chronic allograft nephropathy. We found that ischemia added to the allogeneic background resulted in significant inflammatory injury, clearly activating and accelerating the cellular mechanisms involved in this process. Introducing modifications to the immunosuppressive treatment, we showed that regimes incorporating rapamycin suppressed the inflammatory T-cell-mediated acute cellular changes associated with renal ischemic injury, improved long-term outcome, and attenuated chronic allograft nephropathy.[3]

Dendritic cells have been recently implicated in the coordination of innate and adaptive immune responses due to the different stages in their life history.[5,7] Also, a relationship between upregulation of DC differentiation and ischemic acute renal failure has been described,[7] thus connecting ischemia to adaptive response.[1,4,11–13] Nevertheless, the impact of reduced oxygen availability in DC

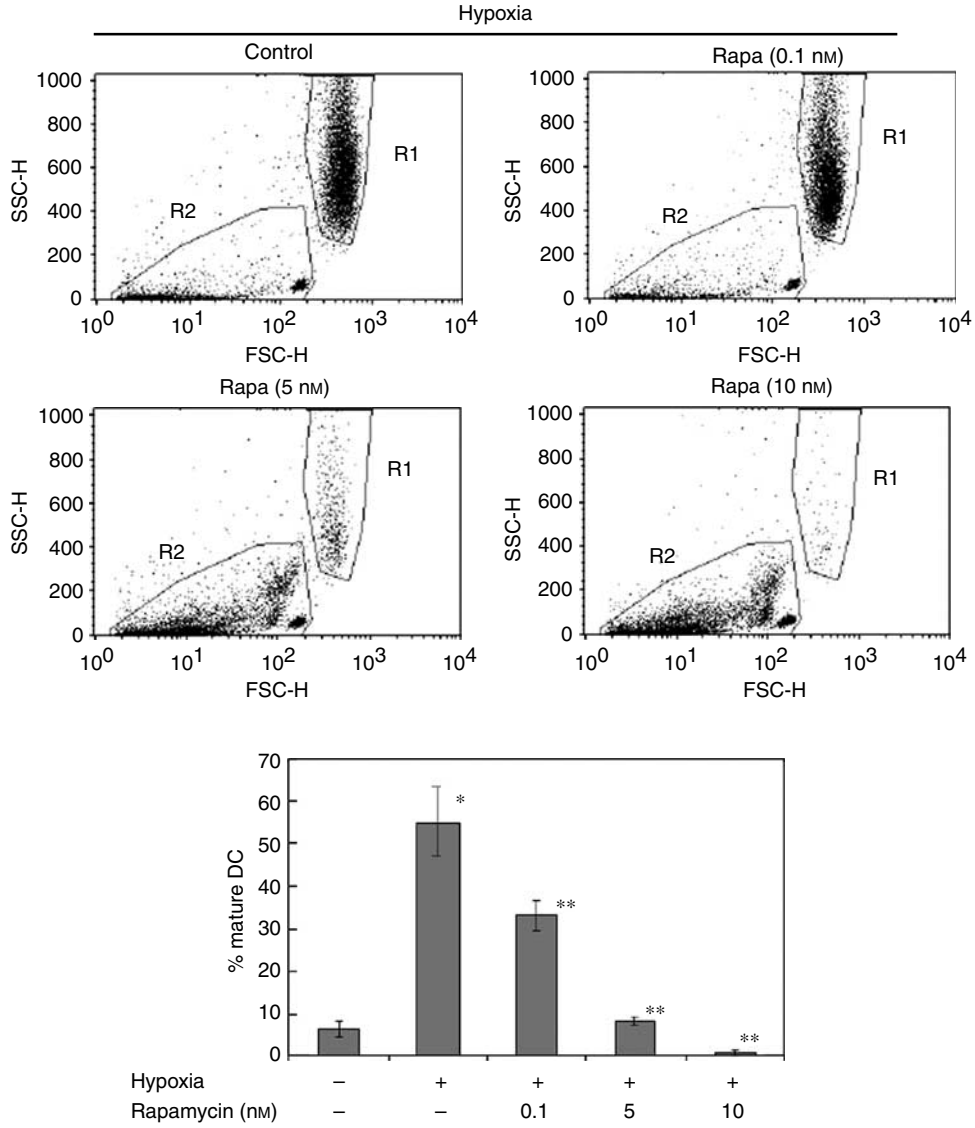

Figure 3 | Rapamycin exerts a dose-dependent effect on hypoxia-stimulated iDCs. Monocytes were cultured with IL-4 and GM-CSF and treated with rapamycin 0.1 nM, 5 nM, and 10 nM. On day 5, iDCs were exposed to 48 h of hypoxia (0.5%). Mean fluorescence intensity of DCs and morphologic changes (FSC-H and SSC-H) were abrogated in a dose-dependent manner by rapamycin treatment. The results are representative of three independent experiments and expressed as the mean (± s.d.) percentage of mature DCs. Hypoxia-stimulated iDCs versus control conditions (no stimuli) (*$P<0.05$). Rapamycin-treated hypoxia-stimulated cells showed a reduction of mature DC forms versus hypoxia-iDCs (**$P<0.05$). Rapa, rapamycin.

differentiation as well as the responsible pathways implicated in this process and whether rapamycin may play a direct role on this effect as we found in our *in vivo* study need to be established.

We first evaluated *in vitro* with DCs, two of the injuries affecting a graft during transplantation, for example, IR and alloreactivity. Although ischemia is a complex process *in vivo*, hypoxia of cell cultures is accepted as a closed environment to study IR. Our findings show that iDCs exposed to hypoxia underwent cell maturation comparable to, and as strong as, alloantigen-dependent maturation. Paradoxically, the combination of the hypoxic and allogeneic stimulus revealed no additive effects. Curiously, iDCs exposed to isogeneic culture alone showed an increase in costimulatory molecular expression. Interestingly, it has been reported that DCs but not macrophages are capable of being activated under isogeneic mixed cultures.[14,15]

To verify whether this maturation in DC phenotype under hypoxia triggers an allostimulatory effect, we analyzed DC cytokine production and the lymphocyte response under mixed culture. High IL-10 secretion was revealed on hypoxia-stimulated DCs. Uniquely among cytokine from hematopoietic cells, IL-10 is a pleiotropic molecule that displays both immunostimulatory and immunoregulatory activities. Although human DCs are generally poor producers of IL-10, it has been shown that high IL-10-producing capacity

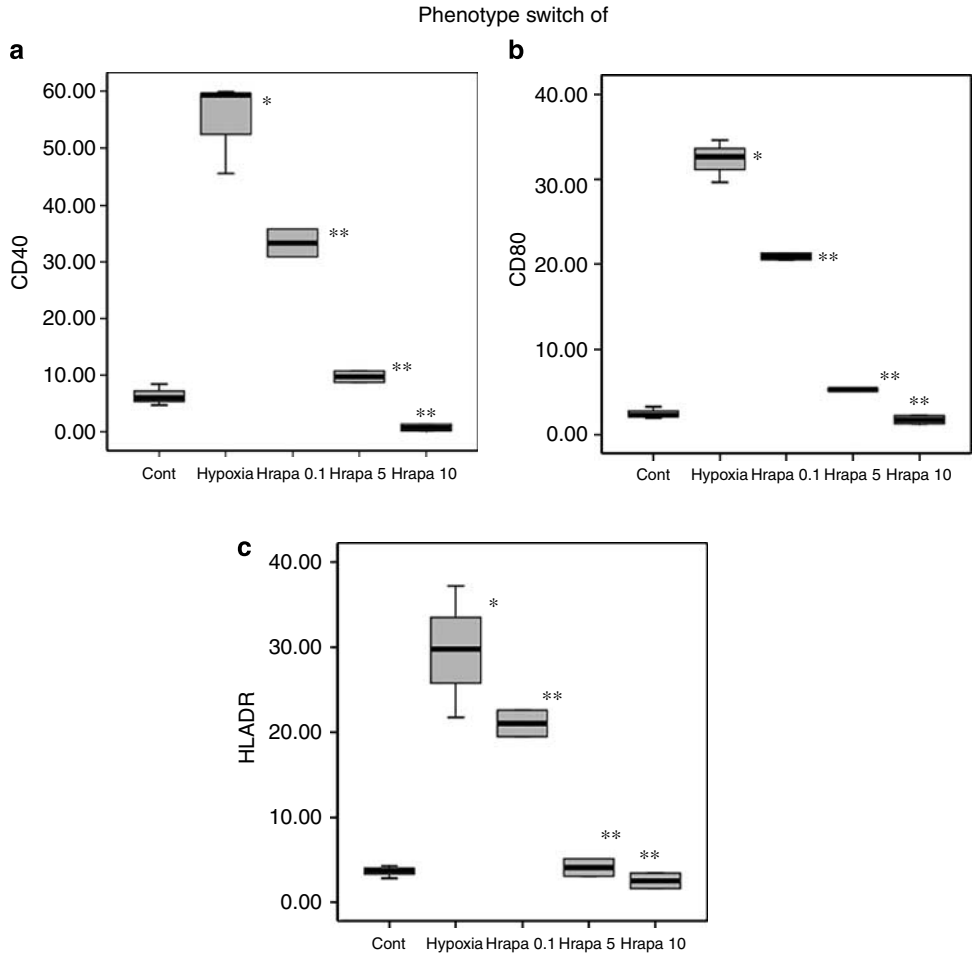

Figure 4 | Hypoxic conditions cause phenotypic and morphologic changes in iDCs. Monocytes were cultured with IL-4 and GM-CSF and treated with rapamycin 0.1 nM, 5 nM, and 10 nM. On day 5, iDCs were exposed to 48 h of hypoxia (0.5%) as described in Materials and Methods. On day 7, cells were harvested and analyzed for CD40, CD80, CD11c, and HLA DR surface markers by flow cytometry. Mean fluorescence intensities of DCs and morphologic changes (FSC-H and SSC-H) were presented as percentage of $CD11c^+$ double marked cells and were markedly higher than under control conditions and comparable to allogeneic exposure. (**a**–**c**) Mean fluorescence intensity of DC surface markers was abrogated in a dose-dependent manner by rapamycin treatment. The results are representative of three experiments. Hypoxia-stimulated iDCs versus control conditions (no stimuli) (*$P<0.05$). Rapamycin-treated hypoxia-stimulated cells showed a reduction of mature DC forms versus hypoxia-iDCs (**$P<0.05$).

can be induced in monocyte-derived DCs by their pretreatment in early stages of maturation with prostaglandin E2 (PGE2).[16] In fact, PGE_2 can modulate the activities of professional DCs by acting on their differentiation and their ability to secrete cytokines.[17] In our results, although there was an upregulation of IFN-γ in hypoxia-stimulated iDCs, usually related to iDC stage maturative change, the IL-10 overproduction was 40% higher. In fact, PGE_2 is associated with suppression of IFN-γ in response to maturation stimuli. It is well-known that hypoxia is associated with increased levels of PGE_2 in several cell lines, enhancing VEGF expression and HIF-1 transcriptional activity by activating the mitogen-activated protein kinase pathway.[18] Interestingly, hypoxia-differentiated DCs provoked a clear proliferation of CD3 lymphocytes similar to allogeneic-differentiated DC exposure. These data confirm that hypoxia-DCs have a functional effect, as they evoke a fully immune response, with lymphocyte expansion.

Definitively, the hypoxia marker genes VEGF and Glut-1 were overexpressed in iDCs exposed to hypoxia, confirming that iDCs followed the classical adaptive hypoxic-induced changes. We also examined HIF-1α, a master regulator molecule under conditions of reduced oxygen availability and classically upregulated by PGE_2. Although extensively studied in macrophages,[19] there are no reports correlating HIF-1α expression and target gene induction with DC maturation. Interestingly, in this study, DC maturation, HIF-1α expression, and HIF-1 target genes expression are interrelated based on their sensitivity to rapamycin. For instance, some studies correlate HIF-1α with cell mediated-inflammation. In fact, the expression of HIF-1α under various immune-inflammatory conditions such as Crohn's disease or rheumatoid arthritis has recently been noted.[20–22] Studies based on the selective deletion of the HIF-1α gene in granulocytes and monocytes have also demonstrated its essential role in myeloid cell mediated-inflammation.[23,24] Our findings in

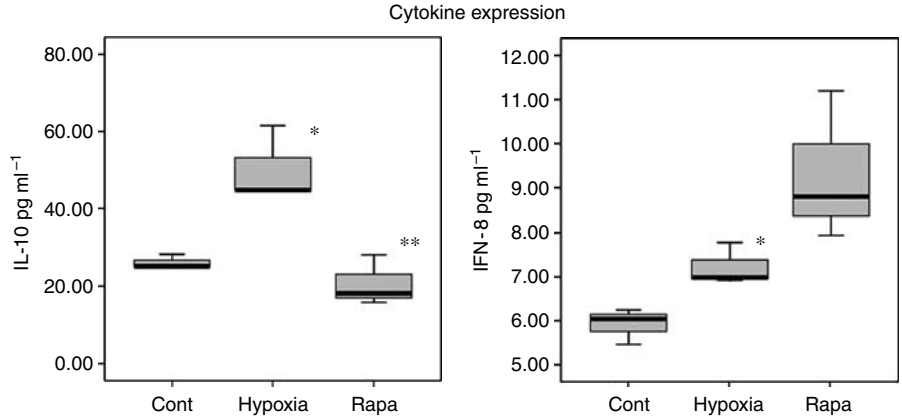

Figure 5 | IL-10 and IFN-γ were oversecreted by hypoxia-stimulated iDCs and IL-10 protein production was downregulated with rapamycin. Monocytes were cultured with IL-4 and GM-CSF (with or without rapamycin (5 nM)). On day 5, iDCs were exposed to 48 h of hypoxia (0.5%) as described in Materials and Methods. On day 7, cells were harvested and analyzed for cytokines secretion (IL-2, IL-4, IL-6, IL-10, TNF, and IFN-γ) by flow cytometry. IL-10 secretion was increased under hypoxia as well as IFN-γ. Nevertheless, IL-10 production was 40% higher than IFN-γ production. IL-10 oversecretion was abrogated by rapamycin treatment (5 nM). The results are representative of three independent experiments and expressed as the geo mean (± s.d.) percentage of cytokine levels. Hypoxia-IL-10 and IFN-γ production versus control conditions (no stimuli) (*$P<0.05$). Hypoxia-stimulated cells treated with rapamycin showed a reduction of IL-10 secretion versus hypoxia-iDCs (**$P<0.05$). Rapa, rapamycin.

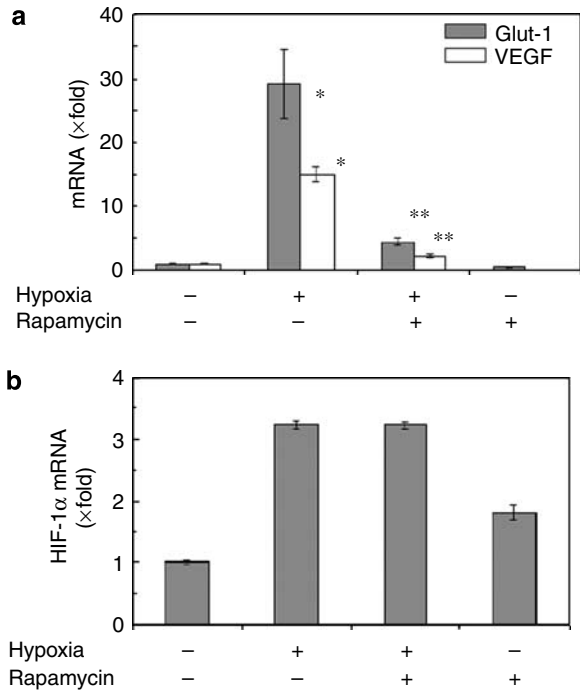

Figure 6 | VEGF, Glut-1 and HiF-1α expression by hypoxia-stimulated iDC. (**a**) VEGF and Glut-1 production by hypoxia-stimulated iDCs or by nonstimulated iDs (control conditions). iDCs were incubated on day 5 in the presence or absence of hypoxic conditions for 48 h. Monocytes were treated (or not) with rapamycin (5 nM). cDNA expression was analyzed by reverse transcription-PCR. VEGF and Glut-1 production by hypoxia-stimulated iDCs versus control conditions (no stimuli) (*$P<0.05$). VEGF and Glut-1 production by hypoxia-stimulated iDCs treated with rapamycin versus hypoxia-iDCs (**$P<0.05$). (**b**) Hypoxia-mediated accumulation of HIF-1α expression in rapamycin-treated (nM) DCs. Human iDCs were incubated in the absence or presence of rapamycin and stimulated (or not) with hypoxia for 48 h. cDNA expression was analyzed by reverse transcription-PCR. No changes in HIF-1α mRNA were found when cells exposed to hypoxia were treated with rapamycin.

DCs concur with those data. Thus, activation of iDCs following hypoxia may trigger a local increase in immunogenicity, which would tether and activate T cells to draw an immune-inflammatory response to the organ. Whether this response is Th1- or Th2-mediated appears an interesting aim for further studies. Our results may indicate that, as a consequence of hypoxia, DC maturation links the innate and the adaptive immune systems, which was previously mainly attributed to T cells.[1]

To further explore the involvement of mTOR pathway in hypoxia related changes in DC stage, we analyzed the behavior of hypoxia-maturated DC vis-à-vis rapamycin. Hypoxia-stimulated iDCs showed a strong downregulation in DC maturation in the presence of rapamycin, an effect that occurred in a dose-dependent manner. Increasing concentrations of rapamycin resulted in the complete inhibition of costimulatory molecular expression. In this sense, Monti *et al.*[25] recently reported that rapamycin, but not calcineurin inhibitors, decreased CD40 expression in differentiated DCs following antigen challenge, leading to the hypothesis that rapamycin was nontoxic. Thus far, our findings demonstrate a critical role of mTOR in the regulation of DC maturation under hypoxia, also defined by Humar *et al.*[26] in smooth muscle cell proliferation.

The inhibitory effect of rapamycin on DC maturation under hypoxia was not associated with a relevant induction of apoptosis. Some discrepancies do exist regarding apoptotic phenomena on rapamycin-treated DCs. In fact, rapamycin has been shown to induce apoptosis in iDCs and CD34-derived DCs but not in monocytes, which exhibit a higher apparent sensitivity to the drug after 2–3 days of culture.[27,28] Nevertheless apoptosis of DCs might also interrupt the effective presentation of antigen and contribute to the prevention of allograft rejection through the efficient inhibition of DC-induced T-cell proliferation.[29]

Rapamycin has been also shown to block the protein synthesis of HIF-1α and its target genes.[30–34] This was confirmed for DCs in our study, where rapamycin decreased HIF-1α expression as well as VEGF and Glut-1 target gene expression. Furthermore and as it was expected, IL-10 secretion was also significantly reduced. In fact, rapamycin inhibits IL-10 production by interfering with the IL-10 gene transcription.[35] Interestingly, CD3 lymphocyte proliferation rate was significantly reduced when CD3 cells were cocultured with rapamycin-treated DCs exposed to hypoxia. Thus, rapamycin, originally known for its interference with cell-cycle progression, also interrupts HIF-1α expression and maturation of DCs under hypoxia and, more important, the consequent lymphocyte expansion.

Recently, novel evidence shows that rDCs participate in the secretion of inflammatory mediators that contribute to early pathophysiological events in the ischemic acutely injured kidney.[10] As a final aim in our studies, we wanted to confirm *in vivo* the differentiation of rDCs after IRI. Thus, kidneys exposed to ischemia showed a clear increase in the number of viable mature rDCs, different from recruited monocytes. In agreement with *in vitro* results, rapamycin treatment abolished this cell differentiation. As it has been said,[10] rDCs in peritubular epithelial space may respond to endogenous activators of innate immunity, to instigate secondary responses in renal epithelial and endothelial cells, and participate in the recruitment of additional circulating cells to the kidney.

In conclusion, this study brings a further approach of the impact of reduced oxygen availability in DCs, thus clarifying the immune inflammatory changes occurring under this condition. Furthermore and as far as we know, mTOR, HIF-1α, and HIF-1 target gene activation are linked for the first time with DC maturation under hypoxic conditions.

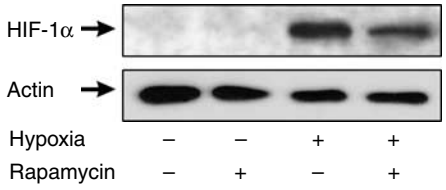

Figure 7 | Hypoxia-mediated accumulation of HIF-1α protein expression in rapamycin-treated (5 nM) DCs. Human iDCs were incubated in the absence or presence of rapamycin and stimulated (or not) with hypoxia for 48 h. Whole cell extracts were prepared and immunoblotting was performed with anti- HIF-1α and β-actin antibodies as described in Materials and Methods.

MATERIALS AND METHODS

Materials for *in vitro* and *in vivo* study

Medium and supplements were purchased from PAA (Linz, Austria). GM-CSF, human IL-4, Annexin-V, desferrioxamine, 7-AAD, and

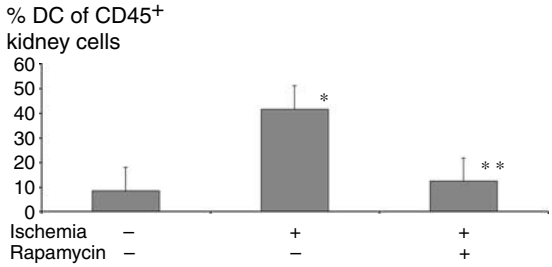

Figure 9 | Ischemia–reperfusion induced rDC maturation. Cell suspensions prepared from the kidney 24 h after 30 min of ischemia, control kidneys, and ischemic kidneys from rapamycin-treated mice (8 mg kg^{-1} for 3 days prior to IRI) were stained for the pan-leukocyte marker CD45 in combination with CD11c, CD80, and either Ly6C or 7-AAD. Results of the overall analysis of cell subsets in ischemic, rapamycin-ischemic, and control kidneys are expressed as percentage of the total CD45 cell suspensions and are representative for a group of four mice for each condition. rDCs within ischemic kidneys exhibited a more mature phenotype than nonischemic kidneys (*$P < 0.05$). Rapamycin-ischemic kidneys showed a reduction of viable mature CD80 rDC forms (**$P < 0.05$), close to control kidneys.

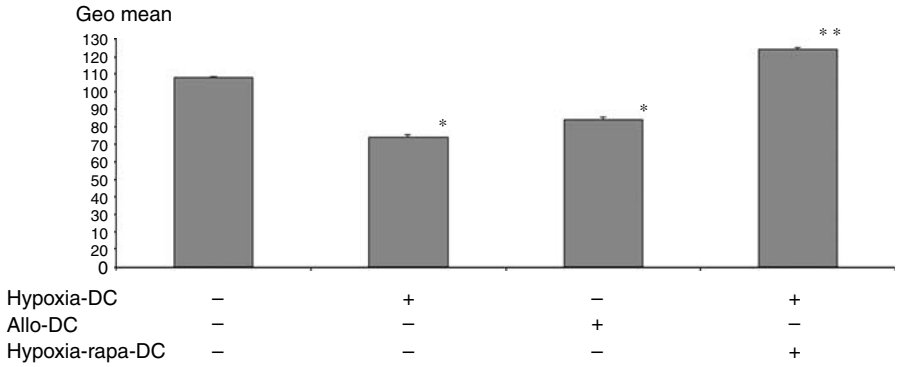

Figure 8 | Lymphocyte proliferation was increased after mature DC exposure. Lymphocytes were CFSE stained and exposed to mature DCs (differentiation under hypoxia (hypoxia-DCs) or allogeneic stimuli (allo-DCs)) or exposed to rapamycin-treated DCs exposed to hypoxia (hypoxia-rapa-DCs) for 24 h. After 10 days, we analyzed by FACS CFSE staining on CD3 cells that were not exposed to DCs (control) and on CD3 cells exposed to DCs. CD3 cells proliferation was significantly higher when those cells were cocultured with mature DCs (differentiated with hypoxia and allogeneic stimuli) (*$P < 0.05$). Interestingly, proliferation rate was significantly reduced when CD3 cells were cocultured with rapamycin treated DC exposed to hypoxia (**$P < 0.05$). The results are representative of three independent experiments and expressed as the geo mean (± s.d.).

rapamycin were ordered from Sigma (Madrid, Spain). A protein assay kit was purchased from Bio-Rad (München, Germany). Anti-human CD3/CD40/CD54/CD58/CD80/CD86/HLA DR/CD11c and CD14 monoclonal antibody were obtained from BD-Pharmingen (Barcelona, Spain) as well as anti-mouse CD45-biotin, CD11c-PE, CD80-FTIC, anti-CD80-fluorescein isothiocyanate isotype control, and LyC6-fluorescein isothiocyanate monoclonal antibodies; and Cytometric Bead Array-CBA, CFSE, and DiOC6 were from Molecular Probes (Madrid, Spain). Nitrocellulose membrane, enhanced chemiluminescence (ECL) detection system, and horseradish peroxidase-labeled anti-mouse or anti-rabbit secondary antibodies were delivered by Amershan Biosciences (Freiburg, Germany). Anti-HIF-1α antibodies and Clontech-Advantage RT-for-PCR kit were purchased from Becton-Dickinson (Heidelberg, Germany). Primers were ordered from MWG-Biotech (Ebersberg, Germany). The peqGOLD-RNAPure kit was acquired from Peqlab (Erlangen, Germany).

Cell culture

Highly enriched monocytes populations (90% $CD14^+$) were obtained from buffy coats of donors through the courtesy of Saarbrücken hospital (Saarbrücken, Germany). Monocytes were obtained by Ficoll–Percoll gradients, purified by adherence, and cultured for 7 days on 6 cm dishes (1×10^6 cells per ml) in RPMI medium supplemented with 2 mM L-glutamine, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 10% fetal calf serum , 5 ng ml^{-1} IL-4 and 100 ng ml^{-1} of GM-CSF, with or without 0.1 nM, 5 nM, or 10 nM of rapamycin being added on day 1. Cells were kept at 37 °C in a humidified atmosphere with 5% CO_2 and propagated according to the protocol provided by the American Type Culture Collection. Medium was changed every second day and prior to experiments with supplements and inhibitors. Lymphocytes were obtained by Ficoll–Percoll gradients, purified by nonadherence, and frozen for subsequent experiments based on isogenic or allogenic mixed culture.

Cell differentiation

Immature DCs were exposed at day 6 of culture for 24 h to lymphocytes (1:10 ratio) from the same donor for isogeneic reactions or to lymphocytes (1:10 ratio) from a different donor for allogeneic reactions. Hypoxic (0.5% oxygen) conditions were generated at day 5, exposing iDCs for 12, 24, and 48 h. iDCs were exposed to hypoxia (0.5% O_2, 5% CO_2) in a hypoxia workstation incubator (*In vivo* 400, Ruskin Technology, UK), thus allowing cell manipulation without O_2 pressure changes.

CD3 lymphocytes were isolated after 24 h of coculture with DCs and proliferation was evaluated by FACS analyze after 10 days of culture.

Quantification of apoptosis and cell proliferation rate

Apoptosis was measured by Annexin-V and also by the loss of mitochondrial membrane potential as detected by decreased emission from the $DiOC_6$. $DiOC_6$ (40 nM) was added 30 min prior to the end of incubations and the green fluorescence of the dye was measured by FACS. Results were expressed as the mean values of DiOC6 staining. Cell proliferation was assessed by CFSE analyze by FACS.

Cell phenotype and cytokine analysis by flow cytometry

Granulocyte–macrophage colony-stimulating factor- and IL-4-treated monocytes (1×10^6 cells per ml) were cultured for 5 days prior to stimulation. Following cell stimulation, cells were washed from the culture dish and transferred to FACS tubes. Thereafter, CD40/CD80/CD86/HLADR/CD54/CD58 and CD11c antibodies were added and incubations continued for 45 min. All incubations were carried out at 4 °C. Flow cytometry analysis was performed using a FACSCalibur flow cytometer (BD Biosciences, Heidelberg, Germany). CD14 expression was assessed by fluorescein isothiocyanate-conjugated anti-CD14 antibodies at days 1 and 5 under resting conditions.

Interleukin-2, -4, -6, -10, TNF-α, and FN-γ secretion protein levels were quantitatively measured following cell stimulation by Cytometric Bead Array System (BD Biosciences, San Diego, CA, USA). The geometric mean values of the log fluorescence in samples were recorded.

RNA extraction, reverse transcription-PCR, and real-time PCR

Total RNA was isolated using the peqGOLD RNAPure kit according to the manufacturer's protocol. Reverse transcription of 1 μg RNA was performed using oligo(dT) primers and MMLV reverse transcriptase. The cDNA was diluted 1:5 for further PCR. For quantitative analysis, equal amounts of 100 ng cDNA were subjected to real-time PCR using the MyiQ Single-Color Real-Time PCR Detection System with SYBR-Green as fluorescent dye. The primer sequences used were as follows: human HIF-1α forward: 5′-CTC AAAGTCGGACAGCCTCA-3′, reverse: 5′-CCCTGCAGTAGGTTT CTGCT-3′; VEGF forward: 5′-TACCTCCACCATGCCAAGTG-3′, reverse: 5′ AAGATGTCCACCAGGGTCTC-3′; Glut-1 forward: TCA CTGTGCTCCTGGTTCTG-3′, reverse 5′- CCTGTGCTCCTGAGAG ATCC-3′; and human RPLO forward: 5′-TGACGGGGTCACCCACA CTGTGCCCATCTA-3′, and reverse: 5′-CTAGAAGCATTTGCGGT GGACGATGGAGGG-3′. For quantification, the expression was normalized to the internal standard gene RPLO.

Western blot analysis

Hypoxia inducible factor-1α and actin were quantified by western analysis. Briefly, cells were scraped off, lysed in 150 μl buffer A (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet-40, 1 mM PMSF, protease inhibitor cocktail, pH 8.0), and sonicated. Following centrifugation (15 000 *g*, 15 min), the protein content in the supernatant was determined, and 100 μg protein was added to the same volume of 2 × SDS-polyacrylamide gel electrophoresis sample buffer (125 mM Tris-HCl, 2% SDS, 10% glycerin, 1 mM DTT, 0.002% bromophenol blue, pH 6.9) and boiled for 5 min. Proteins were resolved on 7.5 or 10% SDS-polyacrylamide gels and blotted onto nitrocellulose membranes. Nonspecific binding was blocked with 5% milk solution in TTBS (50 mM Tris-HCl, 140 mM NaCl, 0.05% Tween 20, pH 7.2) for 1 h. HIF-1α, actin antibodies (1:1000 in 1% milk/TTBS) were added and incubated overnight at 4 °C. Afterwards, nitrocellulose membranes were washed three times for 5 min each with TTBS. Blots were then incubated with goat anti-mouse or goat anti-rabbit secondary antibodies conjugated with peroxidase for 1 h, washed three times for 5 min each with TTBS, followed by ECL detection.

Mouse model of IRI and preparation of kidney cell suspension

Adult C57BL/6 (B6) mice were purchased from Charles River (Bicester, UK), and housed in a specific pathogen-free facility. Unilateral renal artery clipping was carried out for 30 min in adult mice under ketamine and isofluorane anesthesia. Kidneys were harvested after 24 h, dissected, cut into 1–2 mm^3 pieces, placed in

RPMI containing 1.6 mg ml^{-1} collagenase I (Sigma Aldrich, Madrid, Spain), and 200 mg ml^{-1} DNase I (Roche Applied Science, Madrid, Spain) for 40 min at 37 °C, washed, resuspended in RPMI/200 mg ml^{-1} DNase I at room temperature for 15 min, and finally washed twice in RPMI. Following erythrocyte lysis, cells were resuspended in RPMI/10% fetal calf serum. Final kidney cell suspension rested for 20 min to allow sedimentation and subsequently the upper two-thirds removed for flow cytometry.

Statistical analysis

Each experiment was performed at least three times and representative data are shown. Significant differences between samples were determined by one-way analysis of variance followed by Tukey's *post hoc* test with Statistical Package for the Social Sciences (SPSS) statistical package. Data in bar graphs are given as the mean ± s.d. A value of $P<0.05$ was considered significant. Differences in cytokine expression were compared using the coefficient of variation as a quotient of dispersion.

ACKNOWLEDGMENTS

We are indebted to Elke Dauber, Sabine Oldendorf, and Sandra Christmann for their technical contribution. We appreciate Tobias Schmid for his scientific collaboration and Dr Ramón Vilella for his pivotal role in dendritic cell isolation. This research was supported by ISCIII/FIS (PI03/0082), ISCIII/FIS (PI05/1049), ISCIII/FIS (PI06/0067), and MEC (SAF2004-04705). Núria Lloberas is a researcher from Transplant Network. Marcel.la Franquesa holds fellowships from Fundació Catalana de Trasplantament and from PROLIGEN in the FP6-2005-LIFESCIHEALTH-7. Immaculada Herrero-Fresneda, Nuria Lloberas, and Inés Rama are researchers from ISCIII/FIS.

REFERENCES

1. Land WG. The role of postischemic reperfusion injury and other nonantigen-dependent inflammatory pathways in transplantation. *Transplantation* 2005; **79**: 505–514.
2. Herrero-Fresneda I, Torras J, Lloberas N *et al.* Reduction of post-ischemic renal inflammation: an effective strategy to attenuate chronic allograft nephropathy. *Transplantation* 2005; **79**: 165–173.
3. Herrero-Fresneda I, Torras J, Cruzado JM *et al.* Do alloreactivity and cold ischemia cause different elementary lesions on chronic allograft nephropathy? *Am J Pathol* 2003; **162**: 127–137.
4. Kim BS, Lim SW, Li C *et al.* Ischemia-reperfusion injury activates innate immunity in rat kidneys. *Transplantation* 2005; **79**: 1370–1377.
5. Quiroga I, McShane P, Koo DDH *et al.* Major effects of delayed graft function and cold ischaemia time on renal allograft survival. *Nephrol Dial Transplant* 2006; **21**: 1689–1696.
6. Rabb H. The T cell as a bridge between innate and adaptative immune systems: implications for the kidney. *Kidney Int* 2002; **61**: 1935–1946.
7. Wu CJ, Sheu JR, Chen HH *et al.* Modulation of monocyte-derived dendritic cell differentiation is associated with ischemic acute renal failure. *J Surg Res* 2006; **32**: 104–111.
8. Fang Y, Vilella-Bach M, Bachmann R *et al.* Phosphatidic acid-mediated mitogenic activation of mTOR signaling. *Science* 2001; **294**: 1942–1945.
9. Loverre A, Ditonno P, Crovace A *et al.* Ischemia-reperfusion induces glomerular and tubular activation of proinflammatory and antiapoptotic pathways: differential modulation by rapamycin. *J Am Soc Nephrol* 2004; **15**: 2675–2686.
10. Dong X, Swaminathan S, Bachman LA *et al.* Resident dendritic cells are the predominant TNF-secreting cell in early renal ischemia–reperfusion injury. *Kidney Int* 2007; **71**: 619–628.
11. Chakraborti T, Mandal A, Mandal M *et al.* Complement activation in heart diseases. Role of oxidants. *Cell Signal* 2000; **12**: 607–617.
12. Zhai Y, Shen XD, Gao F *et al.* The TLR-4 signaling pathway links innate and adaptative immunity in liver ischemia-reperfusion injury in mice. *J Immunol* 2004; **173**: 7115–7119.
13. Kim BS, Lim SW, Li C *et al.* Ischemia-reperfusion injury activates innate immunity in rat kidneys. *Transplantation* 2005; **79**: 1370–1377.
14. Matsue H, Edelbaum D, Hartmann AC *et al.* Dendritic cells undergo rapid apoptosis *in vitro* during antigen-specific interaction with CD4+ T cells. *J Immunol* 1999; **162**: 5287–5298.
15. Guidos C, Wong M, Lee KC. A comparison of the stimulatory activities of lymphoid dendritic cells and macrophages in T proliferative responses to various antigens. *J Immunol* 1984; **133**: 1179–1184.
16. Kalinski P, Hilkens CM, Snijders A *et al.* IL-12-deficient dendritic cells, generated in the presence of prostaglandin E2, promote type 2 cytokine production in maturing human naive T helper cells. *J Immunol* 1997; **159**: 28–35.
17. Harizi H, Gualde N. Pivotal role of PGE_2 and IL-10 in the cross-regulation of dendritic cell-derived inflammatory mediators. *Cell Mol Immunol* 2006; **3**: 271–277.
18. Kaidi A, Qualtrough D, Williams AC, Paraskeva C. Direct transcriptional up-regulation of cyclooxygenase-2 by hypoxia-inducible factor (HIF)-1 promotes colorectal tumor cell survival and enhances HIF-1 transcriptional activity during hypoxia. *Cancer Res* 2006; **66**: 6683–6691.
19. Schmid T, Zhou J, Bruene B. HIF-1 and p53: communication of transcription factors under hypoxia. *J Cell Mol Med* 2004; **8**: 423–431.
20. Makino Y, Nakamura H, Ikeda E *et al.* Hypoxia-inducible factor regulates survival of antigen receptor-driven T cells. *J Immunol* 2003; **171**: 6534–6540.
21. Giatromanolaki A, Sivridis E, Maltezos E *et al.* Upregulated hypoxia inducible factor-1alpha and -2alpha pathway in rheumatoid arthritis and osteoarthritis. *Arthritis Res Ther* 2003; **5**: R193–R201.
22. Hollander AP, Corke KP, Freemont AJ, Lewis CE. Expression of hypoxia-inducible factor 1alpha by macrophages in the rheumatoid synovium: implications for targeting of therapeutic genes to the inflamed joint. *Arthritis Rheum* 2001; **44**: 1540–1544.
23. Kojima H, Gu H, Nomura S *et al.* Abnormal B lymphocyte development and autoimmunity in hypoxia-inducible factor 1alpha -deficient chimeric mice. *Proc Natl Acad Sci USA* 2002; **99**: 2170–2174.
24. Cramer T, Yamanishi Y, Clausen BE *et al.* HIF-1alpha is essential for myeloid cell-mediated inflammation. *Cell* 2003; **112**: 645–657.
25. Monti P, Mercalli A, Leon Biagio E *et al.* Rapamycin impairs antigen uptake of human dendritic cells. *Transplantation* 2003; **75**: 137–145.
26. Humar R, Kiefer FN, Berns H *et al.* Hypoxia enhances vascular cell proliferation. *Faseb J* 2002; **16**: 771–780.
27. Woltman AM, de Fijter JW, Kamerling SW *et al.* Rapamycin induces apoptosis in monocyte- and CD34-derived dendritic cells but not in monocytes and macrophages. *Blood* 2001; **98**: 174–180.
28. Hackstein H, Taner T, Zahorchak AF *et al.* Rapamycin inhibits IL4-induced dendritic cell maturation *in vitro* and dendritic cell mobilization and function *in vivo*. *Blood* 2003; **101**: 4457–4463.
29. Matsue H, Yang C, Matsue K *et al.* Contrasting impacts of immunosuppressive agents (rapamycin FK506, cyclosporin A, and dexamethasone) on bidirectional dendritic cell-T cell interaction during antigen presentation. *J Immunol* 2002; **169**: 3555–3564.
30. Hidalgo M, Rowinsky EK. The rapamycin-sensitive signal transduction pathway as a target for cancer therapy. *Oncogene* 2000; **19**: 6680–6686.
31. Nakamura H, Makino Y, Okamoto K *et al.* TCR engagement increases hypoxia-inducible factor-1 alpha protein synthesis via rapamycin-sensitive pathway under hypoxic conditions in human peripheral T cells. *J Immunol* 2005; **174**: 7592–7599.
32. Guba M, Graeb C, Jauch KW, Geissler EK. Pro- and anti-cancer effects of immunosuppressive agents used in organ transplantation. *Transplantation* 2004; **77**: 1777–1782.
33. Mayerhofer M, Valent P, Sperr WR *et al.* BCR/ABL induces expression of vascular endothelial growth factor and its transcriptional activator, hypoxia inducible factor-1alpha, through a pathway involving phosphoinositide 3-kinase and the mammalian target of rapamycin. *Blood* 2002; **100**: 3767–3775.
34. Mayerhofer M, Aichberger KJ, Florian S *et al.* Identification of mTOR as a novel bifunctional target in chronic myeloid leukemia: dissection of growth-inhibitory and VEGF-suppressive effects of rapamycin in leukemic cells. *FASEB J* 2005; **19**: 960–962.
35. Jorgensen PF, Wang JE, Almlof M *et al.* Sirolimus interferes with the innate response to bacterial products in human whole blood by attenuation of IL-10 production. *Scand J Immunol* 2001; **53**: 184–191.