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Renal tubular PD-L1 (CD274) suppresses alloreactive human T-cell responses

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Renal proximal tubular epithelial cells, a target of infiltrating T cells during renal allograft rejection, may be protected from this injury by the cell surface protein CD274 (also termed PD-L1 for programmed death ligand 1). The co-inhibitory molecules PD-L1 (CD274) and PD-L2 (CD273) are ligands of PD-1 (programmed death 1; CD279). Here we determine the functional role of PD-1/PD-L pathways in human renal allograft rejection. Treatment of human primary tubular epithelial cells with interferon-β and -γ caused a dose-dependent and synergistic increase of PD-L1 and PD-L2 expression. Blockade of surface PD-L1, but not PD-L2, on interferon-treated tubular epithelial cells resulted in a significant increase in $CD4^+$ T-cell proliferation and cytokine production by $CD4^+$ and $CD8^+$ T cells. The expression of PD-L1, PD-L2, and PD-1 mRNA and protein was upregulated in biopsies of patients with renal allograft rejection compared to the respective levels found in the pre-transplant biopsies. Induction of PD-L1 was significantly associated with acute vascular rejection. Our study suggests that the renal epithelial PD-1/PD-L1 pathway exerts an inhibitory effect of on alloreactive T-cell responses. The upregulation of PD-L1 on proximal tubular epithelial cells in patients with acute allograft rejection may reduce T-cell-mediated injury.

Kidney International (2010) **78,** 38–47; doi:10.1038/ki.2010.97; published online 14 April 2010

KEYWORDS: acute rejection; immunology; renal proximal tubule cell

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Part of this work has been presented as poster and was published in abstract form (*Am J Transplant* 2008; **8**(suppl 2): 418) at the American Transplant Congress 2008 in Toronto.

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Received 4 June 2009; revised 31 December 2009; accepted 9 February 2010; published online 14 April 2010

Acute renal allograft rejection remains a major obstacle for long-term allograft survival as it predisposes to chronic allograft injury with a progressive decline of renal function. Although the various damages to the allograft (including acute or chronic rejection) resulting in interstitial fibrosis/tubular atrophy (IF/TA) are not unequivocal, it is widely accepted that the incidence and severity of acute rejection is associated with chronic organ damage and eventually allograft survival.[1]

Renal proximal tubular epithelial cells (TECs) are the main target of infiltrating alloreactive $CD4^+$ and $CD8^+$ T cells causing tubulointerstitial injury.[2] The local inflammation induces major histocompatibility complex (MHC) class I and class II expression on donor TECs, allowing recognition of allogeneic MHC/peptide complexes by recipient T cells (signal 1).[3] An additional signal 2 required for full T-cell activation is provided by the binding of costimulatory molecules on antigen-presenting cells to their receptors on T cells (for example, B7/CD28). In contrast, a number of known co-inhibitory molecules downregulate T-cell responses. PD-L1 (B7-H1, CD274) and PD-L2 (B7-DC, CD273) are ligands for the programmed death 1 (PD-1) molecule that is induced on activated T and B cells. PD-L1 and PD-L2 are expressed on hematopoietic cells and parenchymal tissues such as heart, lung, liver, placenta, and kidney.[4,5] Binding of PD-L1 or PD-L2 to PD-1 inhibits lymphocyte activation.[4,5]

The expression profile of costimulatory and co-inhibitory molecules on TECs is limited.[6] Although TECs do not express the typical costimulatory molecules B7.1 and B7.2,[7,8] they express CD40 as a costimulatory molecule. PD-L1 is the most prominent co-inhibitory molecule on TECs. Along with others, we previously observed that PD-L1 is strongly expressed in rejected murine kidney transplants, and that PD-L1 is markedly upregulated on cultured murine TECs after treatment with inflammatory cytokines.[9,10] Furthermore, high PD-L1 expression by murine TECs impairs antigen-specific $CD4^+$ and $CD8^+$ T-cell responses.[6,9,11] Several other studies showed that the PD-1/PD-L1 pathway

directly attenuates allograft rejection in experimental models of heart, skin, and pancreas transplantation.[12–14] Furthermore, PD-1 had a crucial role for the induction of $CD8^+$ T-cell tolerance in an allogeneic mixed chimerism model.[15]

The functional role of PD-1/PD-L pathways in human kidney allograft rejection is unknown. Thus, the aim of this study was to investigate the function of PD-L1 and PD-L2 on human TECs in modulating alloreactive $CD4^+$ and $CD8^+$ T-cell responses. Furthermore, expression of PD-L1, PD-L2, and PD-1 was evaluated in biopsies of kidney allografts with acute and chronic rejection.

RESULTS

PD-L1 and PD-L2 surface expression on human renal TECs on IFN-β and IFN-γ treatment *in vitro*

To explore the functional role of PD-L1 and PD-L2 on human TECs, we first studied the expression pattern on cultured TECs. TECs were stimulated with human interferon (IFN)-β and IFN-γ for 48 h. PD-L1 and PD-L2 were constitutively expressed on TECs (Figure 1a). Treatment with either IFN-β or IFN-γ was able to further upregulate the expression of both PD-L1 and PD-L2 in a dose-dependent manner (Figure 1a). Furthermore, stimulation of TECs with both IFNs revealed a synergistic effect (Figure 1b). We performed these experiments with primary TECs ($n = 3$) and HK-2 cells and obtained similar results (data not shown).

Stimulation with IFN-β and IFN-γ also strongly upregulated constitutive MHC class I expression (Figure 1c). MHC class II molecules were not constitutively expressed on TECs (Figure 1c), but stimulation with IFN-γ led to a marked increase. IFN-β had no effect.

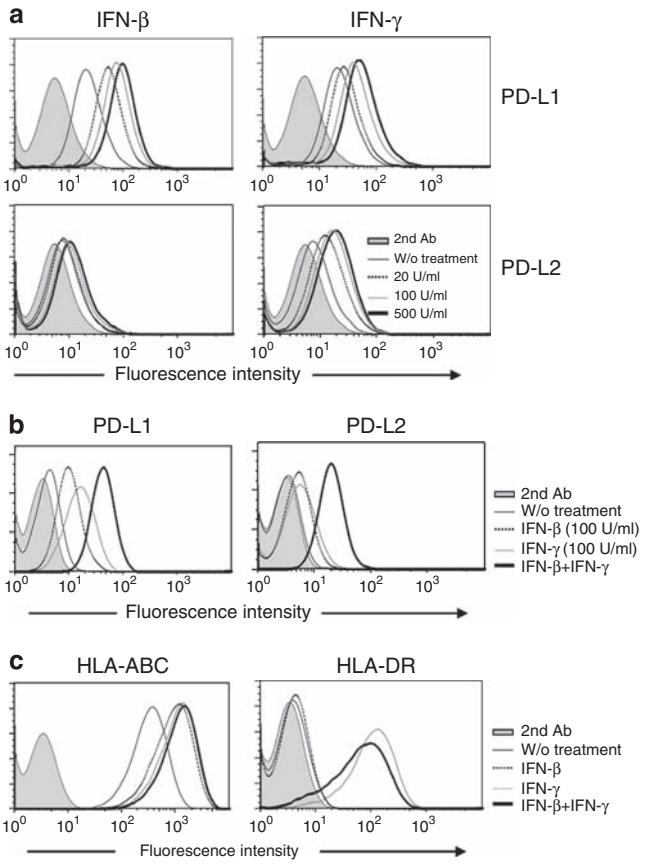

Figure 1 | Flow cytometric analysis of PD-L1/PD-L2 and MHC class I/class II on human primary tubular epithelial cells (TECs). (**a** and **b**) Human primary TECs were stained with monoclonal antibodies for PD-L1 and PD-L2 expression after 48 h stimulation with interferon (IFN)-β or IFN-γ at different doses (**a**) as well as after treatment with both cytokines together (**b**) and compared to untreated controls. (**c**) Major histocompatibility complex (MHC) class I and class II expression was determined with antibodies against HLA-ABC and HLA-DR, respectively. The results are representative of the three patients' TECs as well as the cell line HK-2.

PD-L1 on TECs inhibited proliferation and cytokine production of $CD4^+$ T cells

In these experiments, alloreactive $CD4^+$ T-cell proliferation was performed by incubating PHA-activated human $CD4^+$ T cells with IFN-pretreated human TECs. Anti-PD-L1 and anti-PD-L2 monoclonal antibodies (mAbs) or isotype controls were included to examine the function of renal epithelial PD-L1 and PD-L2. As shown in Figure 2a, compared to the anti-CD3 mAb-induced $CD4^+$ T-cell proliferation, TECs generated from nephrectomized kidneys of two patients (P1 and P2 TEC) induced a very weak T-cell proliferation. Importantly, double blockade of PD-L1 and PD-L2 on both P1 and P2 TECs significantly increased the $CD4^+$ T-cell proliferation and cytokine production (Figure 2a and c). To further define whether the enhancement of T-cell proliferation was mediated by the blockade of PD-L1, PD-L2 or both, we performed single blockade. Interestingly, inhibition of PD-L1 alone was able to augment $CD4^+$ T-cell proliferation, which was comparable to the responses obtained with double blockade. In contrast, PD-L2 blockade was not effective (Figure 2b).

The cytokine profile in the supernatants of $CD4^+$ T cell/TECs cocultures was investigated, including IL-2, IL-4, IL-10, IFN-γ, and tumor necrosis factor (TNF)-α. Similar to the T-cell proliferation, TECs stimulated negligible IFN-γ production comparable to the baseline level of IFN-γ production by $CD4^+$ T cells alone (Figure 2c). Inhibition of PD-L1 on TECs significantly increased the IFN-γ production up to 5- to 10-fold. Again, the same magnitude of IFN-γ secretion was seen when both PD-L1 and PD-L2 on TECs were blocked, whereas inhibition of PD-L2 alone had no effect. TNF-α secretion by $CD4^+$ T cells was also observed only after PD-L1, but not after PD-L2 blockade (data not shown). Although proliferation of $CD4^+$ T cells after blockade of PD-L1 on TECs could be measured, IL-2 production was not detectable in the supernatants of $CD4^+$ T cell/TECs coculture after 72 h (data not shown). In addition, $CD4^+$ T cells did not produce IL-4 or IL-10 after stimulation with TECs under these experimental conditions (data not shown).

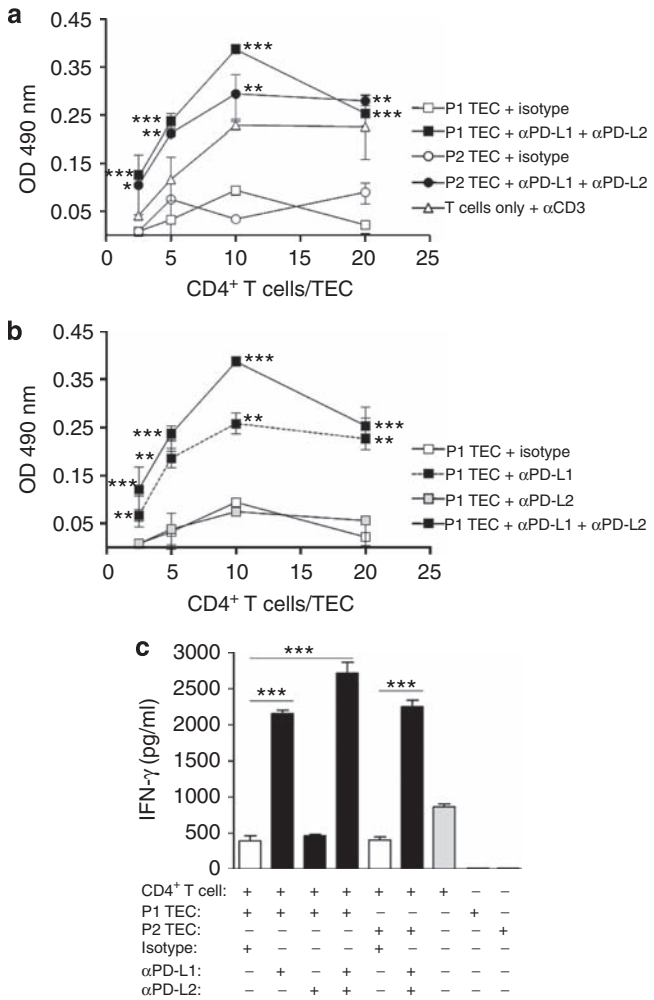

Figure 2 | Proliferation and interferon-γ (IFN-γ) production of CD4$^+$ T cells after stimulation with human primary tubular epithelial cells (TECs) pretreated with IFNs. CD4$^+$ T cells activated with PHA were cocultured with IFN-pretreated TECs of patient 1 (P1 TEC) or of patient 2 (P2 TEC) (2×10^4 cells per well) at the indicated responder/stimulator ratios of 20, 10, 5, and 2.5:1. Before addition of TECs to T cells, TECs were incubated for 1 h with αPD-L1 and/or αPD-L2 antibodies or isotype control. After 72 h of coculture, the proliferation (**a** and **b**) and the IFN-γ production (**c**) of CD4$^+$ T cells were determined. The results represent mean values ± s.d. of triplicate wells and are representative of CD4$^+$ T-cell responses of four different donors that gave similar results. *$P<0.05$, **$P<0.001$, ***$P<0.0001$ when compared with isotype control (unpaired Student's *t*-test).

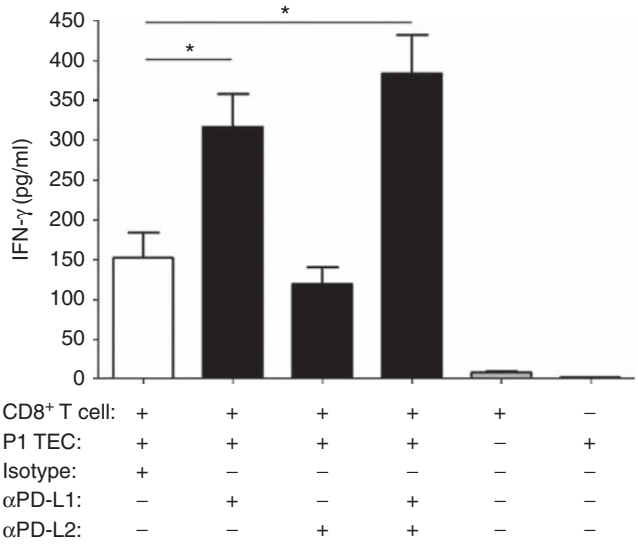

Figure 3 | Interferon-γ (IFN-γ) production of CD8$^+$ T cells after stimulation with human primary tubular epithelial cells (TECs) pretreated with IFNs. CD8$^+$ T cells activated with PHA were cocultured with IFN-pretreated TECs of patient 1 (P1 TEC) (1×10^4 cells per well) at the responder/stimulator ratio of 30:1. Before addition of TECs to CD8$^+$ T cells, TECs were incubated with αPD-L1 and/or αPD-L2 antibodies or isotype control. After 48 h of coculture, the IFN-γ production of CD8$^+$ T cells was determined. The results represent mean values ± s.d. of triplicate wells and are representative of CD8$^+$ T-cell responses of four different responders that gave similar results. *$P<0.05$, when compared with isotype control (unpaired Student's *t*-test).

PD-L1 on TECs inhibited cytokine production of CD8$^+$ T cells

To study the alloreactive CD8$^+$ T-cell response after stimulation with TECs, we used TECs pretreated with IFNs as stimulators to test the IFN-γ production by PHA-activated CD8$^+$ T cells. Similar to the CD4$^+$ T-cell responses, TECs alone only induced weak CD8$^+$ T-cell activation, measured by a low IFN-γ production in the supernatants of T cell/TECs cocultures. Blockade of both PD-L1 and PD-L2 on the TECs significantly increased the IFN-γ production of CD8$^+$ T cells by two- to threefold (Figure 3). In analogy to the CD4$^+$ T-cell responses, single PD-L1 blocking on TECs increased the IFN-γ production of CD8$^+$ T cells to the same magnitude as with the double blocking, and no increase of IFN-γ production was obtained by PD-L2 blocking (Figure 3).

PD-L1 on TECs inhibited the IFN-γ production of alloreactive CD4$^+$ and CD8$^+$ T cells

Because PHA activates T cells nonspecifically and independently of TCR signaling, our results may not be completely transferable to alloreactive T-cell responses. Thus, we repeated these experiments by using allogeneic and autologous pre-stimulation of CD4$^+$ and CD8$^+$ T cells. Autologously and allogeneically pre-stimulated CD4$^+$ and CD8$^+$ T cells were cocultured with IFN pretreated TECs of patient 2. Because blockade of PD-L2 on TECs did not increase the T-cell response in the previous experiments (Figures 2 and 3), only PD-L1 blockade was performed. Allogeneic pre-activation of CD4$^+$ T cells with T-cell-depleted peripheral blood mononuclear cells (PBMCs) of patient 2 induced high amounts of IFN-γ compared to autologous pre-activation of CD4$^+$ T cells (Figure 4a, T cells alone), indicating that the allogeneic pre-stimulation effectively activated CD4$^+$ T cells of the healthy donor.

Restimulation of these allogeneically or autologously pre-activated CD4$^+$ T cells with the TECs of this patient 2 (P2 TEC) could not further increase the IFN-γ secretion. However, inhibition of PD-L1 on P2 TEC significantly augmented the IFN-γ production by allogeneically and to a much lesser degree also by autologously pre-activated CD4$^+$

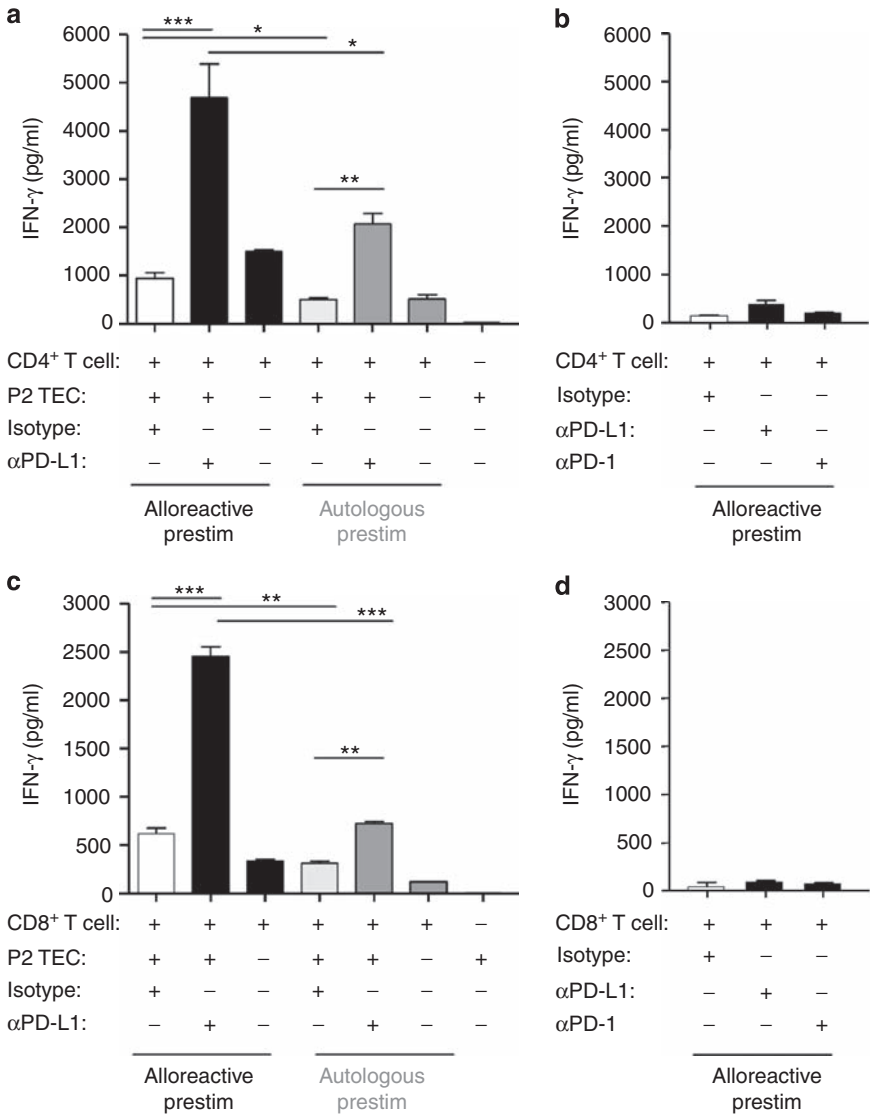

Figure 4 | Interferon-γ (IFN-γ) production of alloreactive CD4$^+$ and CD8$^+$ T cells after stimulation with human primary tubular epithelial cells (TECs) pretreated with IFNs. CD4$^+$ and CD8$^+$ T cells were pre-activated with allogeneic or autologous T-cell-depleted peripheral blood mononuclear cells (PBMCs) and were cocultured with IFN-pretreated TECs of patient 2 (P2 TEC) at a responder/stimulator ratio of 20 and 30 for the CD4$^+$ T cells and the CD8$^+$ T cells, respectively (**a** and **c**). Before addition of TECs to T cells, TECs were incubated for 1 h with αPD-L1 antibodies or isotype control. After 72 h (**a**) or 48 h (**c**) of coculture, the IFN-γ production of CD4$^+$ T cells (**a**) and CD8$^+$ T cells (**c**) was determined. As control, αPD-L1 or αPD-1 antibodies were also added to the alloreactive CD4$^+$ and CD8$^+$ T-cell cultures in the absence of renal TECs, respectively, for determining IFN-γ production in the supernatants (**b** and **d**). *$P<0.05$, **$P<0.001$, ***$P<0.0001$ when compared with isotype control (unpaired Student's *t*-test).

T cells. As expected, the cytokine response of the allogeneically pre-activated CD4$^+$ T cells was significantly higher.

The alloreactive CD8$^+$ T-cell response showed a similar pattern (Figure 4c). Pre-activation of CD8$^+$ T cells by allogeneic CD4$^-$CD8$^-$ PBMCs of patient 2 induced moderate IFN-γ production. Inhibition of PD-L1 on P2 TEC dramatically increased the IFN-γ secretion of allogeneically pre-activated CD8$^+$ T cells. In contrast, blockade of PD-L1 on P2 TEC only slightly enhanced the IFN-γ production of autologously pre-activated CD8$^+$ T cells. In conclusion, these data reveal that CD4$^+$ and CD8$^+$ T cells pre-activated with the patient's allogeneic CD4$^-$CD8$^-$ PBMCs showed a much stronger cytokine response to P2 TECs than autologously pre-activated T cells.

It is known that PD-1 and PD-L1 are also expressed on activated CD4$^+$ and CD8$^+$ T cells. To rule out the possibility that blockade of PD-L1 on alloreactive T cells alone would increase IFN-γ production of those T cells, we added anti-PD-L1 Abs to the alloreactive pre-stimulated CD4$^+$ and CD8$^+$ T cells, and measured the IFN-γ production in the supernatants. Although the inhibition of PD-L1 on alloreactive CD4$^+$ T cells alone slightly increased the IFN-γ secretion, this was negligible compared to the results obtained from the stimulation of the same CD4$^+$ T cells

together with TECs in the presence of anti-PD-L1 blocking Abs (Figure 4b). Similarly, blockade of PD-L1 on $CD8^+$ T cells alone did not increase the IFN-γ production in the supernatants (Figure 4d). In addition, blockade of the receptor PD-1 on both alloreactive $CD4^+$ and $CD8^+$ T cells with mAbs did not increase the IFN-γ production (Figure 4b and d). Thus, our results suggest that PD-L1 on TECs has a predominant role in inhibiting alloreactive $CD4^+$ and $CD8^+$ T-cell responses induced by TECs.

Strong induction of PD-L1, PD-L2, and PD-1 mRNAs in human biopsies of kidney allograft rejection

The mRNA expression of PD-L1, PD-L2, and their receptor PD-1 was then investigated in renal biopsies of patients with acute kidney transplant rejection (ATR), IF/TA,[16] and BK virus nephropathy (BKN) by real-time RT-PCR. Compared to control tissues (pretransplant biopsies; living donor $n=9$, deceased donor $n=1$), PD-L1 mRNA transcript levels were significantly increased only in renal biopsies of patients with ATR, but not in biopsy samples from patients with IF/TA or BKN (Figure 5a). PD-L2 mRNA was significantly enhanced in biopsies of patients with ATR or IF/TA (Figure 5b). In addition, biopsies of all patients showing a high PD-L1 mRNA induction had also increased mRNA levels of PD-L2 (Pearson's correlation, $r=0.6480$, $P=0.0006$). PD-1 mRNA was also significantly induced in biopsies of patients with ATR, IF/TA, and BKN (Figure 5c).

Patient characteristics and correlation to the PD-L1, PD-L2, and PD-1 mRNA levels

The relevant clinical parameters including recipient age, gender, creatinine, and proteinuria as well as histological parameters such as scores for tubulitis, interstitial infiltration, chronic lesions, and Banff classification at the time of biopsy were extracted from the European Renal cDNA Bank/ Kroener-Fresenius Biopsy Bank (ERCB-KFB) (Table 1). PD-L1, PD-L2, and PD-1 mRNA revealed no correlation to creatinine and proteinuria levels. Possibly the cohort size was too small for such analysis. Because follow-up data are not routinely collected from allograft recipients in the ERCB-KFB, a correlation with the response to treatment could not be assessed. However, the expression of PD-L1 was significantly increased in patients with vascular rejection (Banff IIA and IIB) compared to tubulointerstitial rejection (Banff IA and IB) (Figure 6a). This was not the case for PD-L2 (data not shown). In contrast, the score for tubulitis showed a tendency to be lower in patients with vascular rejection (Banff IIA and IIB, Figure 6b).

PD-L1 and PD-1 localization in human allograft rejection

We speculated that the increased mRNA expression of PD-1 detected in the biopsies might be due to infiltrating lymphocytes and the enhanced PD-L1 mRNA expression would be expected on TECs as well as on infiltrating leukocytes. To localize PD-L1 and PD-1, we performed immunohistochemistry on a series of archival allograft

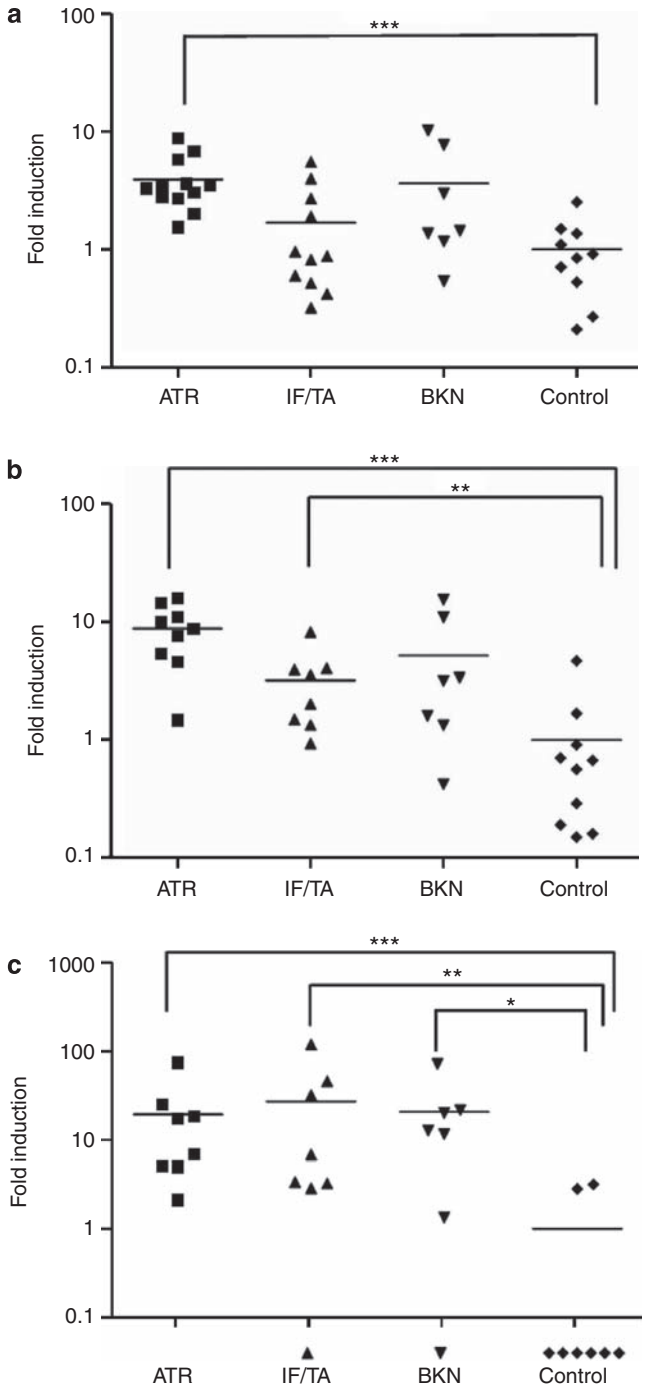

Figure 5 | PD-L1, PD-L2, and PD-1 mRNA expression in human renal biopsies. (**a**) Renal biopsies from patients with acute transplant rejection (ATR, $n=12$), interstitial fibrosis and tubular atrophy (IF/TA $n=11$), and BK virus nephropathy (BKN, $n=7$) were compared to pre-transplant tissue samples (control, $n=10$) for the mRNA expression of PD-L1. (**b**) For PD-L2, 9 samples of ATR, 8 of IF/TA, 7 of BKN, and 10 of controls and (**c**) for PD-1, 8 samples of ATR, 8 of IF/TA, 7 of BKN, and 8 of controls were analyzed. mRNA expression of PD-L1, PD-L2, and PD-1 were normalized to the expression of 18S rRNA and the fold inductions of the target genes in the above-mentioned entities were calculated compared to the control tissue (=1). PD-1 mRNA expression was not detectable in some samples that are shown below the *x* axis. $^*P<0.05$, $^{**}P<0.001$, $^{***}P<0.0001$ when compared with controls (Mann–Whitney *U*-test and Bonferroni correction).

Table 1 | Clinical and histological characteristics of biopsies from patients with acute transplant rejection, interstitial fibrosis/tubular atrophy, BK virus nephropathy, and control subjects analyzed by real-time RT-PCR

						Grade of damage			
Biopsy group	Age (year)	Gender	Graft	Creatinine (μmol/l)	Proteinuria (g per day)	ci/ct	int inf	tub inf	Banff
Acute transplant rejection									
ATR1	47	M	dcd	424	NA	2	3	2–3	IIB
ATR2	71	F	dcd	455	0.5	2	1–2	0	IIA
ATR3	47	M	dcd	240	0.1	0	2	2	IIA
ATR4	49	M	dcd	720	NA	0	2	1	IIA
ATR5	61	F	dcd	456	NA	0	2	3	IB
ATR6	54	F	dcd	909	NA	0	2	2	IA
ATR7	49	M	dcd	177	0.18	2	NA	NA	IA
ATR8	47	M	dcd	440	0.3	0–1	2	3	IB
ATR9	65	M	dcd	488	NA	2	2	2	IIA
ATR10	61	F	dcd	637	NA	1	2	1	IIB
ATR11	43	M	dcd	908	NA	0	2	1	IIB
ATR12	49	M	NA	203	NA	0	1	2	IIA
Interstitial fibrosis/tubular atrophy									
IF/TA1	46	M	dcd	174	NA	1	0–1	0	
IF/TA2	60	F	NA	220	0.325	1	0–1	0	
IF/TA3	23	F	liv	188	NA	1	0–1	0	
IF/TA4	66	F	dcd	120	2	1–2	0	0	[a]
IF/TA5	32	F	dcd	234	NA	3	1	0	
IF/TA6	52	NA	NA	548	1.82	2	0–1	0	[a]
IF/TA7	37	M	NA	884	7	2	0	0	
IF/TA8	32	M	dcd	171	1.5	1–2	0–1	0	[a]
IF/TA9	34	F	dcd	495	0.8	3	0–1	0	
IF/TA10	67	M	dcd	294	0.2	1	0–1	0	
IF/TA11	66	M	dcd	225	5	1	0–1	0	
BK virus nephropathy									
BKN1	37	M	dcd	301	NA	0–1	2	0	
BKN2	41	M	dcd	539	0.4	2	1–2	3	
BKN3	52	F	dcd	273	8.3	0	2	2	
BKN4	35	F	dcd	528	1.26	2	1	0	[a]
BKN5	57	M	dcd	426	NA	1	2	2	
BKN6	66	F	dcd	320	0	3	1–2	0	
BKN7	40	F	dcd	280	2.3	2	1–2	0–1	
Control subjects									
C1	35	F	liv	<97	<0.2				
C2	39	M	liv	<97	<0.2				
C3	55	F	liv	<97	<0.2				
C4	41	M	liv	<97	<0.2				
C5	61	M	liv	<97	<0.2				
C6	58	F	liv	<97	<0.2				
C7	27	M	liv	<97	<0.2				
C8	NA	NA	dcd	<97	<0.2				
C9	54	F	liv	<97	<0.2				
C10	61	F	liv	<97	<0.2				

Banff IA/B: tubulointerstitial rejection; Banff IIA/B: vascular rejection; ci/ct: chronic interstitial fibrosis and chronic tubular atrophy; dcd: deceased donor; int inf: interstitial infiltration/inflammation; tub inf: tubular infiltration/tubulitis; grade of damage/score 0: none or minimal, <5% for ci/ct and <10% for int inf; 1: mild (<25%); 2: moderate <50%; 3: severe >50%; for tub inf 0: no cells/tubular cross section; 1: 1–4 cells per tubular cross section; 2: 5–10 cells per tubular cross section; 3: >10 cells per tubular cross section; liv: living donor; NA: not available.
[a]Transplant glomerulopathy.

biopsies and on two renal allograft nephrectomies with severe acute vascular rejection in addition to signs of chronic injury (IF/TA, $n=2$). The allograft biopsies included acute interstitial rejection (Banff IB) and biopsies without acute rejection with well-preserved tissue architecture ($n=3$). In allograft nephrectomies, surface expression of PD-L1 was found on infiltrating cells in the tubulointerstitium that displayed dendritic cell morphology (Figure 7a) and on TECs (Figure 7a and c). Preincubation of the anti-PD-L1 antibody with its peptide completely abolished the positive PD-L1 staining (Figure 7b). PD-1 was expressed by interstitial infiltrating lymphocytes but not on tubular cells (Figure 7d).

DISCUSSION

The wide distribution of PD-L1 and PD-L2 in lymphoid and parenchymal tissues[4,5]suggests a broad regulatory function on various immune responses, including autoimmunity and self-tolerance.[14] Recent studies indicated an important role

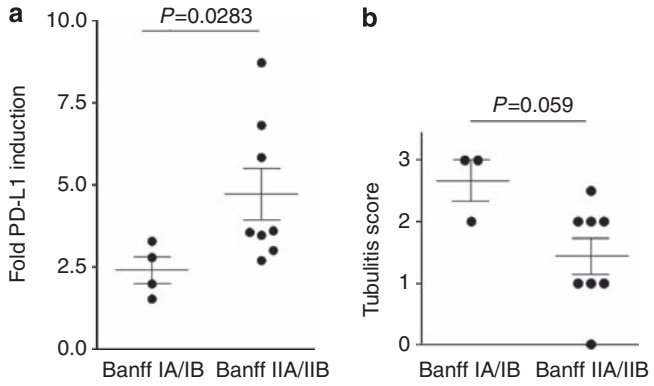

Figure 6 | PD-L1 mRNA induction is pronounced in vascular allograft rejection. (**a**) Relation of PD-L1 induction between patients suffering from acute tubulointerstitial rejection (Banff IA/IB) and vascular rejection (Banff IIA/IIB). (**b**) Comparison of the tubulitis score between tubulointerstitial rejection (Banff IA/IB) and vascular rejection (Banff IIA/IIB) (Mann–Whitney *U*-test).

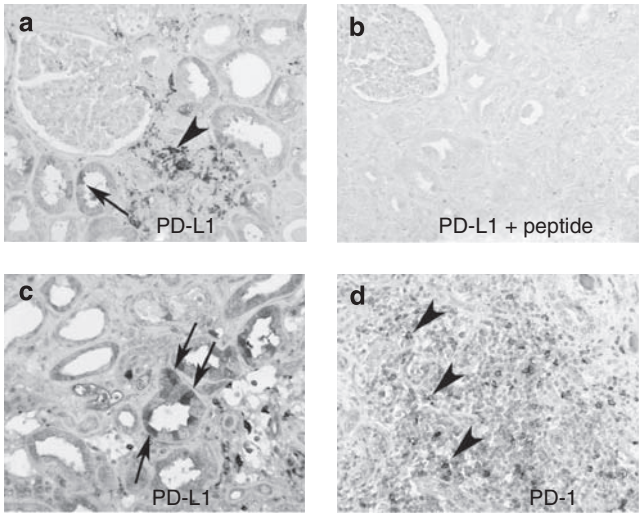

Figure 7 | Immunohistochemistry of PD-L1 and PD-1 expression on allograft biopsies. (**a**) In allograft nephrectomies PD-L1 was expressed by large cells with a dendritic cell morphology in the tubulointerstitium (arrowheads) as well as by some tubular epithelial cells (arrows). (**b**) Preincubation of the anti-PD-L1 antibody with the peptide used for immunization completely abolished the positive staining showing specificity of the staining. (**c**) Prominent expression of PD-L1 by tubular epithelial cells (arrows). (**d**) PD-1-positive cells resemble small lymphocytes (some are indicated by arrowheads), which were present in foci of smaller and larger accumulations of infiltrating cells (original magnifications: $\times$ 100 **a** and **b**; $\times$ 200 **c** and **d**).

for the PD-1/PD-L pathway for allograft rejection and tolerance in experimental models.[12,17] Here, we showed that the co-inhibitory molecule PD-L1 expressed on human renal TECs suppresses human alloreactive T-cell responses and is associated with acute kidney allograft rejection.

Earlier studies showed that $CD4^+$ T cells do not respond to allogeneic TECs pretreated with IFN-γ. This was in part due to the lack of costimulation, especially the absence of B7.1 molecules on TECs.[7,18] However, our study now reveals that the existence of the PD-1/PD-L1 co-inhibitory pathway has a crucial role for the unresponsiveness of alloreactive T cells toward renal TECs, because inhibition of PD-L1 on TECs strongly augmented the $CD4^+$ and $CD8^+$ T-cell responses. An increased alloreactive $CD8^+$ T-cell response could only be measured in terms of IFN-γ production, whereas the same $CD8^+$ T cells were not able to lyse allogeneic renal TECs even though they showed a strong cytotoxic T-lymphocyte (CTL) activity to Jurkat cells (data not shown). These results indicate that human TECs are highly resistant to the attack of alloreactive CTLs *in vitro*. The limited costimulatory molecule expression on TECs, including the lack of strong B7/CD28 costimulation, could at least partially explain this phenomenon.

Our results showed that IFN-β and IFN-γ have a critical role in the upregulation of the PD-L1 and PD-L2 expression on TECs *in vitro*. Obata *et al.*[19] showed that the expression of IFN-γ mRNA was strongly increased in acute and chronic human renal allograft biopsies. It is therefore possible that, during local inflammation, IFN-β production as part of the innate immune response and IFN-γ secretion from infiltrating alloreactive T cells are both responsible for the *in vivo* increase of PD-L1 (and PD-L2) expression on renal tubulointerstitial tissues as observed in our study. This is consistent with the observation that the expression of PD-1 is absent in healthy kidneys without inflammation.[20] The expression of PD-1 in allograft biopsies with acute rejection indicates the presence of activated T cells within the inflamed kidneys. Importantly, more intense tubulitis seems to be associated with lower PD-L1 expression during tubulointerstitial rejection, whereas the opposite was observed for vascular rejections. This may suggest that during more severe acute vascular rejections the tubulointerstitium is partially protected by the expression of PD-L1, whereas this is not the case for the vascular endothelium that does not express PD-L1 (Figure 7a). These results complement previous observations of high PD-L1 expression on renal tubules in other immune-mediated human renal diseases, including diffuse proliferative lupus nephritis, IgA nephropathy, and tubulointerstitial nephritis.[10,20] Therefore there is increasing evidence that the PD-1/PD-L1 pathway is involved in immune-mediated human renal diseases and especially in ATR.

It has been reported that PD-1 blockade reverses the suppression of cancer-specific CTLs by $CD4^+CD25^{hi}$ Tregs.[21] Stroopinsky *et al.*[22] have shown that $CD4^+CD25^{hi}FoxP3^+$ T cells (Tregs) were induced by allogeneic stimulation of PBMC. This allogeneic stimulation induced human $FoxP3^+IFN\text{-}\gamma^+$ T cells that markedly inhibit alloreactive T-cell expansion. Considering the fact that Tregs express high PD-1, one hypothesis is that the blockade of PD-L1 on TECs in our experiments may not only inhibit the PD-1/PD-L1 pathway between alloreactive effector T cells and TECs, but also abolish the PD-1/PD-L1 interaction between Tregs and TECs. This might lead to the impairment of Treg function, hence increasing the effector T-cell responses and resulting in a higher IFN-γ secretion. Further studies on the functional

role of the PD-1/PD-L1 pathway between Tregs and renal TECs would be required to clarify this point. However, in our experiments using isolated $CD8^+$ T cells, $CD4^+CD25^{hi}FoxP3^+$ Tregs were removed by the isolation procedure, therefore making a direct effect on effector cells much more likely.

Although PD-L2 upregulation was observed in our biopsy samples and on IFN-stimulated TECs, it does not seem to have an inhibitory role in human kidney transplantation. There was no correlation to histological parameters and *in vitro* blockade of PD-L2 on TECs was not effective. This was not due to the failure of the anti-human PD-L2 mAb included in this study because the same clone of mAb was successfully used to inhibit PD-L2 activity of T-cell stimulator cells by others.[23] Several studies suggested distinct roles of PD-L1 and PD-L2 in regulating T-cell activity. Tanaka *et al.*[24] indicated that blockade of PD-L1, but not of PD-L2, accelerated cardiac allograft rejection associated with an increased frequency of IFN-γ-producing alloreactive T cells and the expansion of effector $CD8^+$ T cells. Consistent with this observation, Sandner *et al.*[13] showed that inhibition of PD-L1, but not of PD-L2, enhanced $CD4^+$ T-cell activation in a skin graft model. The function of PD-L2 may also depend on the location of immune responses. Habicht *et al.*[25] showed that in the host lymphoid organs as opposed to parenchymal tissue the *in vivo* activation of both alloreactive $CD4^+$ and $CD8^+$ T cells was primarily mediated by PD-L2. Furthermore, the increased proliferation of $CD8^+$ T cells after *in vivo* blockade of PD-L2 was dependent on CD28-mediated costimulation signal,[25] which does not have a role for TECs that do not express B7 molecules.

Experimental models of heart[12,17] and corneal allografts[26–29] showed a critical and protective role of the PD-1/PD-L1 pathway in transplantation. Although the increase of PD-L1 on donor TECs is not sufficient to completely prevent acute allograft rejection, our results suggest a protective mechanism of parenchymal tissues to mitigate alloreactive human T-cell responses and tissue injury through PD-1/PD-L1 co-inhibitory pathway. Strategies to selectively enhance PD-L1 expression on TECs as well as on professional antigen-presenting cells might be therapeutically useful to prevent ATR.

MATERIALS AND METHODS

General reagents

Cell culture reagents were obtained from Invitrogen (Gaithersburg, MD, USA) and Sigma (St Louis, MO, USA). Human cytokines were purchased from R&D Systems (Oxford, UK).

Human cell lines and primary TEC cultures

The human renal TEC line HK-2 was cultured in K1 medium.[30] Primary cultures of human TECs were generated from healthy parts of tumor nephrectomies. Briefly, small fragments of cortical tissue were digested twice with type II collagenase (1 mg/ml in HBSS) (Invitrogen) for 90 min at 37 °C. The cell suspension was passed through 40 μm sieves, washed, and seeded on collagen-coated cell culture plates in K1 medium containing human epidermal growth factor (5 ng/ml). Next day, after washing with HBSS, fresh K1 medium was added to allow primary TECs growing at 37 °C in 5% CO_2 until confluence (5–7 days). Epithelial cell origin confirmed by positive cytokeratin staining was more than 95%. In functional assays, the second passage of TECs with positive cytokeratin expression was used as antigen-presenting cells, which showed a similar pattern of surface molecule expression and antigen-presentation capacity compared to the original primary TECs.

Flow cytometric analysis

Human TECs were harvested by trypsinization, washed, and stained with mAbs (all mouse anti-human) against PD-L1 (clone MIH1), PD-L2 (clone MIH18), HLA-ABC (clone W6/32), and HLA-DR (clone LN3). mAbs were purchased from eBioscience (San Diego, CA, USA). Cells were analyzed using a FACSCantoII flow cytometer (BD Biosciences, San Jose, CA, USA) and FlowJo software Version 7 (TreeStar, Ashland, OR, USA).

Isolation and activation of human $CD4^+$ and $CD8^+$ T cells *in vitro*

Human PBMCs were isolated from heparinized whole blood of healthy volunteers ($n = 5$) and of patient 2 (who provided the tissue for isolating TECs) using Lymphoprep (Axis-Shield PoC AS, Oslo, Norway) according to the manufacturer's protocol. PBMCs were washed with phosphate-buffered saline three times followed by $CD4^+$ and $CD8^+$ T-cell isolation with human CD8 and CD4 MACS microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), respectively. $CD4^+$ and $CD8^+$ T cells were then pre-activated either with PHA or allogeneic PBMCs to upregulate PD-1 expression on T-cell surfaces.

PHA activation. Isolated $CD4^+$ and $CD8^+$ T cells of healthy donors were adjusted to 2×10^6 cells per ml in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum, seeded into 24-well plates and incubated at 37 °C, 5% CO_2. Activation of T cells was performed by adding 2 μg/ml of PHA for 5 days.

Allogeneic and autologous activation. The T-cell-depleted fraction of PBMCs ($CD4^-CD8^-$ PBMCs) of patient 2 was irradiated with 30 Gy and then cocultured with purified $CD4^+$ or $CD8^+$ T cells of one fully HLA-mismatched healthy donor (see below) at a ratio of 2:1 (T cells/PBMCs) for 3 days in the presence of human IL-2 (5 ng/ml). As a control, $CD4^+$ and $CD8^+$ T cells of the same healthy donor were also stimulated with their own $CD4^-CD8^-$ PBMCs (autologous stimulation) under the same conditions. Low-resolution HLA typing of A, B, DR, and DQ loci using PCR-SSP method (PCR with sequence-specific primers) of the responders and patients presented in Figures 2–4 was performed and is indicated below:

- Responder 1: A*02, A*24, B*51, B*57, DRB1*01, DRB1*07, DQB1*05, DQB1*0303
- Responder 2: A*11, A*23, B*44, B*40, DRB1*07, DRB1*09, DRB4*, DQB1*02, DQB1*0303
- Patient 1: A*02, A*02, B*35, B*50, DRB1*07, DRB1*07, DRB4*, DQB1*02, DQB1*02
- Patient 2: A*01, A*26, B*07, B*35, DRB1*11, DRB1*15, DRB3*, DRB5*, DQB1*0301, DQB1*06

For all responder-stimulator combinations tested, we found HLA mismatches in MHC class I and class II loci.

Alloreactive CD4$^+$ T-cell proliferation induced by TECs

Human primary TECs were pretreated with human IFN-β and IFN-γ (100 U/ml each) for 2 days. Cells were then harvested by trypsinization, washed, and irradiated with 60 Gy. Irradiation did not influence the expression of PD-L1 and PD-L2 (data not shown). After washing, we resuspended TECs in Iscove's modified Dulbecco's medium and seeded them in 96-well plates. TECs were preincubated with the relevant mAbs (PD-L1, PD-L2, or isotype control) for 1 h at 37 °C before addition of pre-activated CD4$^+$ T cells at the indicated T cell/TEC ratios. T-cell proliferation was determined after 72 h of co-incubation by using the nonradioactive colorimetric CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. Results were presented as the value of the OD at 490 nm after subtraction of the OD values from medium alone and calculated as follows: $OD_{experimental} - OD_{TEC\ alone} - OD_{T\ cell\ alone}$.

Alloreactive CD8$^+$ T-cell cytokine production stimulated by TECs

Human primary TECs were pretreated with IFN-β and IFN-γ for 2 days, harvested, and preincubated with the relevant mAbs or isotype control for 1 h, and then seeded in 96-well plates. Pre-activated human CD8$^+$ T cells were added at a responder/stimulator ratio of 30:1 (3×10^5 CD8$^+$ T cells/1×10^4 TECs) for 48 h.

Measurement of cytokines

Cytokine production was determined in the coculture supernatants after 48 h (CD8$^+$ T-cell response) and 72 h (CD4$^+$ T-cell response), respectively, either by human-specific enzyme-linked immunosorbent assay kits for IFN-γ or IL-2 (eBioscience) or by the BD CBA Flex Sets (BD Biosciences) according to the manufacturer's protocols.

Quantitative real-time PCR of renal biopsies

Human renal biopsy specimens were procured in an international multicenter study, the ERCB-KFB. Biopsies were obtained from patients after informed consent and with approval of the local ethics committees. Clinical characteristics of all patients are shown in Table 1. Tubulointerstitial tissue was prepared and analyzed by real-time RT-PCR as reported earlier.[31] Predeveloped TaqMan reagents were used for human PD-L1 (CD274) (NM_014143.2), PD-L2 (CD273, PDCD1LG2) (NM_025239.2), and PD-1 (PDCD1, CD279) (NM_005018.1) as well as the housekeeper genes (*GAPDH* and *18SrRNA*; Applied Biosystems, Foster City, CA, USA). The mRNA expression was analyzed by standard curve quantification. Normalization to both housekeeper genes gave comparable results; data based on 18S rRNA are shown.

Immunohistochemistry

Immunohistochemistry was performed as previously described.[32] The biopsies were archival materials collected retrospectively at the University of Vienna (2006–2008). In brief, sections were dewaxed in xylene, rehydrated in a series of graded ethanols, and incubated in 3% hydrogen peroxide (to block endogenous peroxidases). To block endogenous biotin, we used the Avidin/Biotin blocking Kit (Vector, Burlingame, CA, USA). Antigen retrieval was performed in an autoclave oven in antigen retrieval solution. Incubation with the primary antibody was performed for 1 h or overnight. Incubation with biotinylated secondary antibodies was for 30 min, followed by the ABC reagent. For PD-1, we used two consecutive secondary antibodies to enhance the signal. 3,3′-Diaminobenzidine (Sigma, Taufkirchen, Germany) with metal enhancement (resulting in a black colored product) served as detection system. The rabbit polyclonal antibody against PD-L1 (ab41890; Abcam, Cambridge, UK) was used in a dilution of 0.5 μg/ml (1:400 in 10% nonfat dry milk). The antibody was raised against a peptide corresponding to amino acids 144–291 of human PD-L1. The peptide was used to block the signal by preincubation of the antibody as additional control. Furthermore, nonimmune rabbit serum was used as a negative control. The monoclonal mouse anti-human PD-1 antibody (clone MIH4; NatuTec, Frankfurt am Main, Germany) was used in a dilution of 40 μg/ml. Isotype matched mouse IgG1 was used as negative control.

Statistics

All *in vitro* cell experiments were performed in triplicates and were repeated at least twice. Data are expressed as mean ± s.d. The unpaired Student's *t*-test was used to analyze all data of the *in vitro* experiments. Mann–Whitney test was used to perform statistical analysis of PD-L1, PD-L2, and PD-1 mRNA expression in human biopsies. Additional Bonferroni correction was applied to assess for multiple testing for the mRNA levels. Pearson's correlation was conducted to correlate mRNA expressions to the clinical characteristics. Significance was accepted at $P \leqslant 0.05$.

DISCLOSURE

All the authors declared no competing interests.

ACKNOWLEDGMENTS

We thank Stefanie Gaiser for technical assistance with the real-time RT-PCR and Gunther Boysen (Department of Pathology, University Hospital Zürich, Zürich, Switzerland) for his help with the cytokeratin staining of human primary TECs. The allograft biopsies for the immunohistochmistry were kindly provided by Heinz Regele (Clinical Institute of Pathology, University of Vienna, Vienna, Austria). We thank all participating centers of the European Renal cDNA Bank/Kroener-Fresenius Biopsy Bank (ERCB-KFB) and their patients for their cooperation. Active members at the time of the study: CD Cohen, H Schmid, M Fischereder, L Weber, M Kretzler, D Schlöndorff, Munich; JD Sraer, P Ronco, Paris; MP Rastaldi, GD'Amico, Milano; P Doran, H Brady, Dublin; D Mönks, C Wanner, Würzburg; AJ Rees, Aberdeen; F Strutz, GA Müller, Göttingen; P Mertens, J Floege, Aachen; N Braun, T Risler, Tübingen; L Gesualdo, FP Schena, Bari; J Gerth, G Wolf, Jena; R Oberbauer, D Kerjaschki, Vienna; B Banas, BK Krämer, Regensburg; M Saleem, Bristol; H-P Marti, RP Wüthrich, Zürich; W Samtleben, Munich; H Peters, HH Neumayer, Berlin; M Daha, Leiden; C Blume, B Grabensee, Düsseldorf; F Mampaso (deceased), Madrid; J Oh, F Schaefer, M Zeier, H-J Gröne, Heidelberg; P Gross, Dresden; G Tonolo; Sassari; V Tesar, Prague; H Rupprecht, Bayreuth.

Funding sources: This study was supported by the Swiss National Science Foundation grants 3200BO-118202 and 3200BO-105849 to YWM and RPW, by the Hartmann Müller-Foundation to YWM, by the DFG (Se 888/4-1) to SS, by the Else-Kröner-Fresenius Foundation to CDC, and by the Swiss National Science Foundation grant 32003B-129710 to SS.

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