

# Lipopolysaccharide-pretreated plasmacytoid dendritic cells ameliorate experimental chronic kidney disease

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Plasmacytoid dendritic cells play important roles in inducing immune tolerance, preventing allograft rejection, and regulating immune responses in both autoimmune disease and graft-versus-host disease. In order to evaluate a possible protective effect of plasmacytoid dendritic cells against renal inflammation and injury, we purified these cells from mouse spleens and adoptively transferred lipopolysaccharide (LPS)-treated cells, modified *ex vivo*, into mice with adriamycin nephropathy. These LPS-treated cells localized to the kidney cortex and the lymph nodes draining the kidney, and protected the kidney from injury during adriamycin nephropathy. Glomerulosclerosis, tubular atrophy, interstitial expansion, proteinuria, and creatinine clearance were significantly reduced in mice with adriamycin nephropathy subsequently treated with LPS-activated plasmacytoid dendritic cells as compared to the kidney injury in mice given naive plasmacytoid dendritic cells. In addition, LPS-pretreated cells, but not naive plasmacytoid dendritic cells, convert CD4<sup>+</sup>CD25<sup>-</sup> T cells into Foxp3<sup>+</sup> regulatory T cells and suppress the proinflammatory cytokine production of endogenous renal macrophages. This may explain their ability to protect against renal injury in adriamycin nephropathy.

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Plasmacytoid dendritic cells (pDCs) were identified in 1958 as a cell type similar to plasma cells but lacking B cell and plasma cell markers. These cells were found in the T-cell zones of human lymphoid tissue.<sup>1</sup> It has since been recognized that these cells correspond to circulating immature CD11c<sup>-</sup> dendritic cells (DCs) that can be induced to develop DC morphology.<sup>2</sup> The murine equivalent of human pDCs are CD11c<sup>+</sup>CD11b<sup>-</sup>B220<sup>+</sup>Gr-1<sup>+</sup> cells that are present within lymph nodes and spleen, and differ from myeloid dendritic cells (mDCs) that are CD11b<sup>+</sup>B220<sup>-</sup>. Besides these differences in phenotypic markers, pDCs and mDCs also differ from each other in terms of function. mDCs are professional antigen-presenting cells involved in T-cell activation and initiation of adaptive immune response, whereas pDCs are considered to be poor antigen-presenting cells and T-cell stimulators based on their low surface expression of major histocompatibility complex and classical costimulatory molecules. However, upon maturation with CD154 and Toll-like receptors (TLRs), pDCs can become potent antigen-presenting cells and are capable of stimulating T-cell proliferation and differentiation.<sup>3</sup>

DCs can be licensed to become regulatory by a variety of cytokines including interleukin-10 (IL-10)/transforming growth factor- $\beta$  (TGF- $\beta$ ),<sup>4</sup> vasoactive intestinal peptide,<sup>5</sup> and endogenous glycan-binding protein (galectin 1).<sup>6</sup> pDCs are able to induce either T helper (Th)1- or Th2-type immune responses depending on the type of stimulation and the dose of antigen.<sup>7</sup> Upon viral infection, pDCs produce large amounts of type I interferon and have therefore been regarded as an important component of innate immunity.<sup>8</sup> On the other hand, IL-3- and CD154-treated pDCs preferentially prime Th2 cells, possibly through engagement of costimulatory molecules such as OX40 ligand.<sup>9</sup> pDCs can also be modified into both inflammatory and regulatory phenotypes by synthetic oligodeoxynucleotide containing unmethylated cytosine-guanine (CpG), and have been shown to induce allogeneic T-cell hyporesponsiveness and prolong cardiac graft survival by increasing expression of IL-10, TGF- $\beta$ , and IL-6.<sup>10</sup> Zwiorek *et al.*<sup>11</sup> showed that CpG-stimulated pDCs can exhibit proinflammatory effects via

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their expression of IL-1, tumor necrosis factor- $\alpha$ , and interferon- $\gamma$ . In contrast, pDCs modulated by lipopolysaccharide (LPS) have been shown to be capable of suppressing experimental autoimmune encephalomyelitis<sup>12</sup> via enhanced secretion of anti-inflammatory cytokines IL-10, TGF- $\beta$ , and IL-13. Moreover human pDCs stimulated with CpG oligodeoxynucleotide have been shown to play important regulatory roles in autoimmune diseases and graft-versus-host diseases.<sup>13–18</sup> pDCs may also exert effects on regulatory T cells (Tregs); a possible mediator of this effect of pDCs on Tregs is indoleamine 2,3-dioxygenase (IDO).<sup>19,20</sup> Whereas there is an increasing understanding of pDCs, their role in chronic renal disease remains poorly defined.

Adriamycin nephropathy (AN) is a chronic kidney disease model characterized by proteinuria, progressive glomerulosclerosis, and tubulointerstitial damage, and it mimics human focal segmental glomerulosclerosis. Our previous studies have shown that reconstitution of Tregs expressing high levels of Foxp3 (forkhead box P3) in severe combined immunodeficient (SCID) mice with AN reduced glomerulosclerosis and tubular injury in comparison with control SCID mice with AN.<sup>21</sup> Our studies have also shown that macrophages modulated by IL-10/TGF- $\beta$  or IL-13/4 are able to reduce renal injury in murine AN.<sup>22,23</sup> In this study, we sought to determine whether or not adoptive transfer of splenic pDCs that were modified *ex vivo* to a regulatory phenotype and infused into AN mice could protect mice against progressive renal injury. Our results showed that adoptive transfer of pDCs modulated with LPS can in fact protect mice against AN, and suggested two novel mechanisms of protection. An increase in Foxp3+ Tregs by pDCs was observed and the induction of Tregs was found to be IDO dependent. In addition, proinflammatory cytokine production by endogenous renal macrophages was decreased in mice treated with LPS-pretreated pDCs.

## RESULTS

### pDC phenotype and function

To characterize the phenotype of pDCs, the expression of the B7-H family of molecules in naive pDCs and LPS-pretreated pDCs was assessed. LPS-pretreated pDCs expressed higher levels of B7-H1 but not B7-H2, B7-H3, or B7-H4 than did naive pDCs (Figure 1; B7-H1 expression relative to  $\beta$ -actin, naive pDCs vs. LPS-pretreated pDCs  $1.5 \pm 0.3$  vs.  $13.2 \pm 2.7$ ,  $P < 0.01$ ). In addition, to determine the function of pDCs, the cytokine expression of pDCs was assessed; there was stronger expression of IL-10, TGF- $\beta$ , and IDO but not IL-12 in LPS-pretreated pDCs and CpG-pretreated pDCs compared with naive pDCs (IDO mRNA expression relative to  $\beta$ -actin naive pDC vs. LPS-pretreated pDC  $1.8 \pm 0.4$  vs.  $7.2 \pm 1.6$ ,  $P < 0.01$ ). Also, the protein level of IL-10 and TGF- $\beta$  of supernatant and cellular IDO from cultured LPS-pretreated pDCs was much higher than those from cultured naive pDCs ( $P < 0.01$ ; Figure 1d).

### pDCs expressed TLR4 after LPS exposure

As TLR4 functions as a LPS-sensing receptor, we tested TLR4 expression and found that TLR4 was expressed on

significantly more LPS-pretreated pDCs than naive pDCs ( $0.6 \pm 0.1\%$  vs.  $18.2 \pm 2.7\%$ ,  $P < 0.01$ ; Figure 2).

### Effect of pDCs on renal structural injury

At 4 weeks after adriamycin, we examined the effect of pDCs on renal structural injury. In AN, renal injury is characterized by glomerulosclerosis, tubular atrophy, interstitial volume expansion, and interstitial fibrosis. Glomerulosclerosis was significantly reduced in AN mice transfused with LPS-pretreated pDCs compared with AN mice transfused with naive pDCs and untransfused AN mice (Figure 3). Similarly, tubulointerstitial injury was significantly improved in AN mice transfused with LPS-pretreated pDCs compared with mice transfused with naive pDC and untransfused AN mice (Figure 3; normal vs. AN control vs. AN + naive pDC vs. AN + LPS-pretreated pDC: glomerulosclerosis  $2.3 \pm 0.3$  vs.  $28.0 \pm 4.6$  vs.  $28.4 \pm 3.7$  vs.  $12.6 \pm 1.8\%$ ,  $P < 0.01$ , tubular cell height  $7.3 \pm 0.4$  vs.  $1.5 \pm 0.2$  vs.  $1.1 \pm 0.3$  vs.  $5.7 \pm 0.9 \mu\text{m}$ ,  $P < 0.05$ , interstitial volume  $1.8 \pm 0.6$  vs.  $26.8 \pm 1.1$  vs.  $28.3 \pm 2.5$  vs.  $12.6 \pm 1.0\%$ ,  $P < 0.01$ , interstitial fibrosis  $2.3 \pm 0.6$  vs.  $20.1 \pm 0.5$  vs.  $18.9 \pm 1.4$  vs.  $12.3 \pm 1.0\%$ ,  $P < 0.01$ ). These results indicate that LPS-pretreated pDCs protected against renal injury in AN.

### Effect of pDCs on renal function

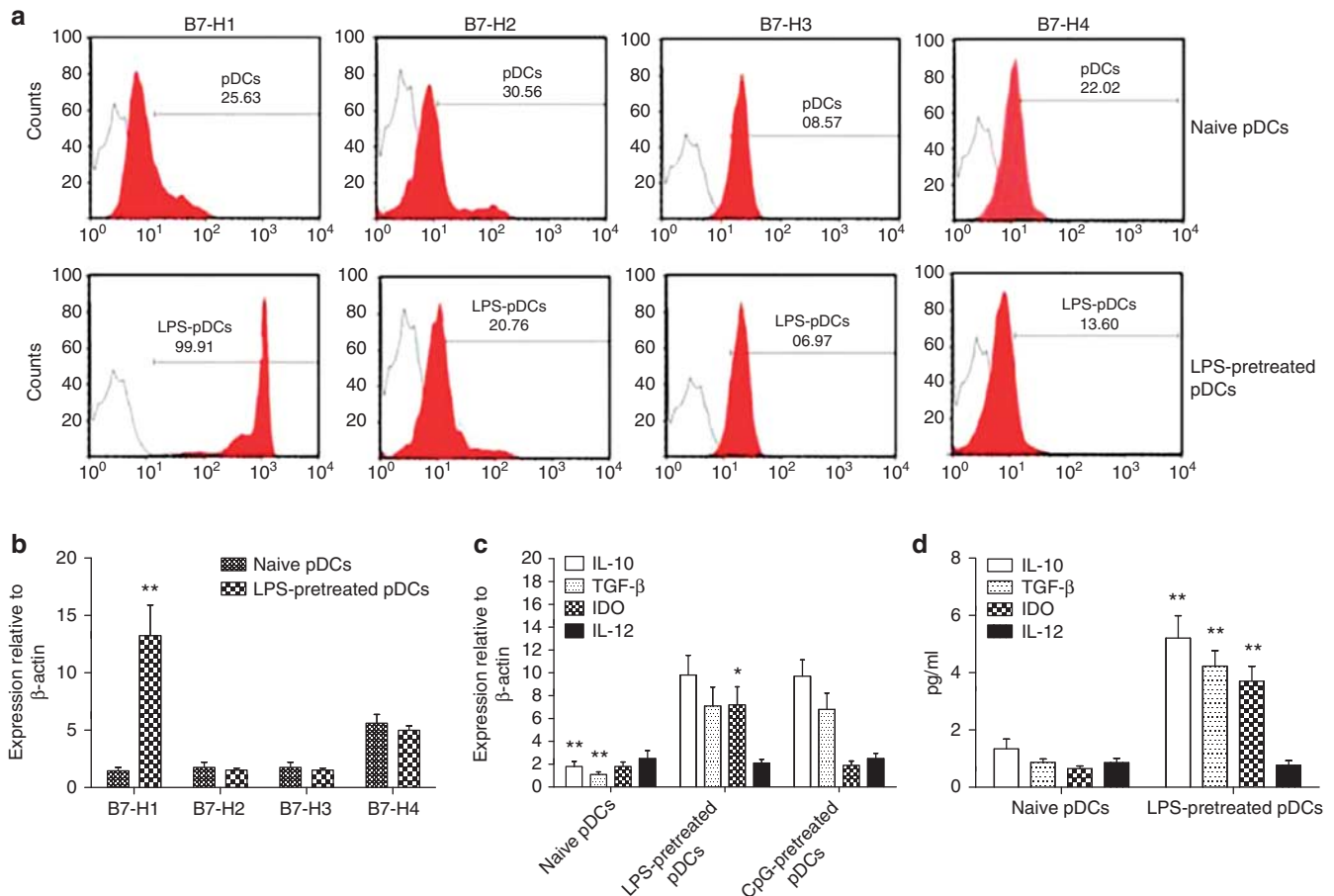
To explore the effect of pDCs on renal function, serum creatinine and urinary protein were measured in each group at 4 weeks after adriamycin. In AN mice, serum creatinine and urinary protein excretion were significantly increased compared with normal mice, and were significantly improved in AN mice transfused with LPS-pretreated pDCs compared with untransfused AN mice or those transfused with naive pDCs. Creatinine clearance was also improved after pDC transfusion (normal vs. AN vs. AN + naive pDC vs. AN + LPS-pretreated pDC: serum creatinine  $5.1 \pm 0.5$  vs.  $16.5 \pm 2.3$  vs.  $18.7 \pm 3.8$  vs.  $11.5 \pm 1.4 \mu\text{mol/l}$ ,  $P < 0.05$ ; urinary protein  $50.7 \pm 26.3$  vs.  $403.5 \pm 104.3$  vs.  $458.8 \pm 49.2$  vs.  $134.5 \pm 23.6 \mu\text{g}/12 \text{h}$ ,  $P < 0.01$ ; creatinine clearance  $34.0 \pm 4.1$  vs.  $14.6 \pm 1.5$  vs.  $15.1 \pm 2.6$  vs.  $24.5 \pm 3.0 \mu\text{l}/\text{min}$ ,  $P < 0.05$ ; urine protein/urine creatinine ratio  $20.8 \pm 2.6$  vs.  $938.4 \pm 98.4$  vs.  $1068.5 \pm 123.4$  vs.  $551.1 \pm 76.2 \mu\text{g}/\text{mmol}$ ,  $P < 0.01$ ; Figure 3).

### Leukocyte infiltration

In AN, focal segmental glomerulosclerosis is accompanied by interstitial inflammation. Therefore, renal leukocyte infiltration at week 4 after adriamycin was examined using anti-F4/80, -CD4, and -CD8 antibodies. The numbers of macrophages, CD4+ T cells, and CD8+ T cells were increased in all AN mice. However, the numbers of these cells were markedly reduced in AN mice transfused with LPS-pretreated pDCs compared with untransfused AN mice and AN mice transfused with naive pDCs (Figure 4).

### pDC tracking in lymph nodes and kidneys

To determine whether a difference in access to organs of interest explained the effects of LPS-pretreated vs. naive



**Figure 1 | Phenotypic features of plasmacytoid dendritic cells (pDCs).** (a, b) The expression of B7-H1, B7-H2, B7-H3, and B7-H4 on naive pDCs and lipopolysaccharide (LPS)-pretreated pDCs was examined by (a) fluorescence-activated cell sorting (FACS) and (b) real-time PCR. (c) The mRNA expression of interleukin-10 (IL-10), transforming growth factor-β (TGF-β), indoleamine 2,3-dioxygenase (IDO), and IL-12 by naive pDCs, LPS-pretreated pDCs, and cytosine-guanine (CpG)-pretreated pDCs was examined by real-time PCR. (d) The protein level of IL-10, TGF-β, IDO, and IL-12 secreted by naive pDCs and LPS-pretreated pDCs was examined by enzyme-linked immunosorbent assay (ELISA). Data represent the mean ± s.d. of three experiments. \* $P < 0.05$  and \*\* $P < 0.01$  vs. counterparts from other groups.

pDCs, tracking studies were performed. At day 28, fluorescently labeled cells were seen in kidney, kidney-draining lymph nodes (KDLNs), and spleen of AN mice transfused with LPS-pretreated pDCs and naive pDCs. Both DiI-stained LPS-pretreated pDCs and naive pDCs were located mostly in kidney interstitium and in paracortex of KDLNs in similar numbers (Figure 5). Thus, the protective effect of LPS-pretreated pDCs vs. naive pDCs was not explained by a difference in access.

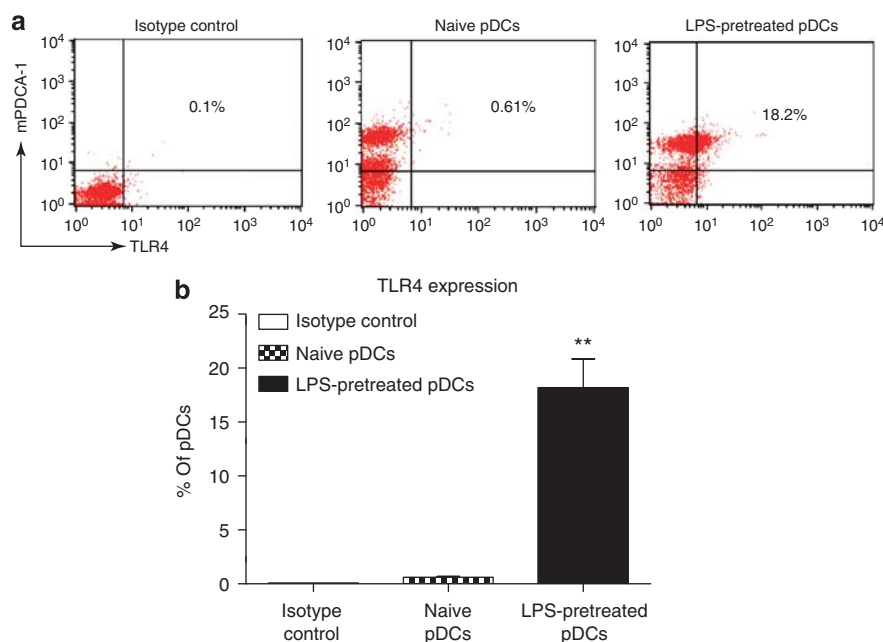
### Induction of Tregs

To further explore the mechanism by which LPS-pretreated pDCs was protective in AN, the number of Foxp3+ CD4+ cells was examined and found to be significantly increased in KDLNs and kidney of AN mice transfused with LPS-pretreated pDCs compared with those treated with naive pDCs or untransfused AN mice (naive pDCs vs. LPS-pretreated pDCs in KDLNs  $6.67 \pm 0.8\%$  vs.  $18.69 \pm 2.5\%$ ,  $P < 0.01$ ; Figure 6a and b). *In vitro*, LPS-pretreated pDCs significantly increased

the percentage of Foxp3+ T cells when cocultured with CD4+ CD25- T cells for 7 days, whereas naive pDCs had no effect. This effect was found to be contact dependent in Transwell experiments (Figure 6c and d). The blockade of IDO expression on modified DCs with 1-methyl-D-tryptophan (D-1MT) eliminated induction of Foxp3+ T cells by LPS-pretreated pDCs, whereas blockade with antibodies against IL-10, TGF-β, or B7-H1 had no effect (Figure 6e). This suggested that at least part of the protective effect of LPS-pretreated pDCs in AN could be due to induction of Tregs in KDLNs and kidney in an IDO-dependent manner.

### Deactivation of effector macrophages *in vitro* and macrophage phenotype *in vivo*

In addition to induction of Foxp3+ Tregs, we explored other potential protective mechanisms by examining the interaction of pDCs with macrophages both *in vitro* and *in vivo*. LPS-pretreated pDCs suppressed proinflammatory cytokine production of effector macrophages (M1) *in vitro* and *in vivo*



**Figure 2 | The expression of Toll-like receptor 4 (TLR4) on plasmacytoid dendritic cells (pDCs).** (a) TLR4 expression on isotype control, naive pDCs, and lipopolysaccharide (LPS)-pretreated pDCs by flow cytometry. (b) Quantitation of percentage of TLR4 expression on cells. Data represent the mean  $\pm$  s.d. of three experiments. \*\* $P < 0.01$  vs. naive pDCs.

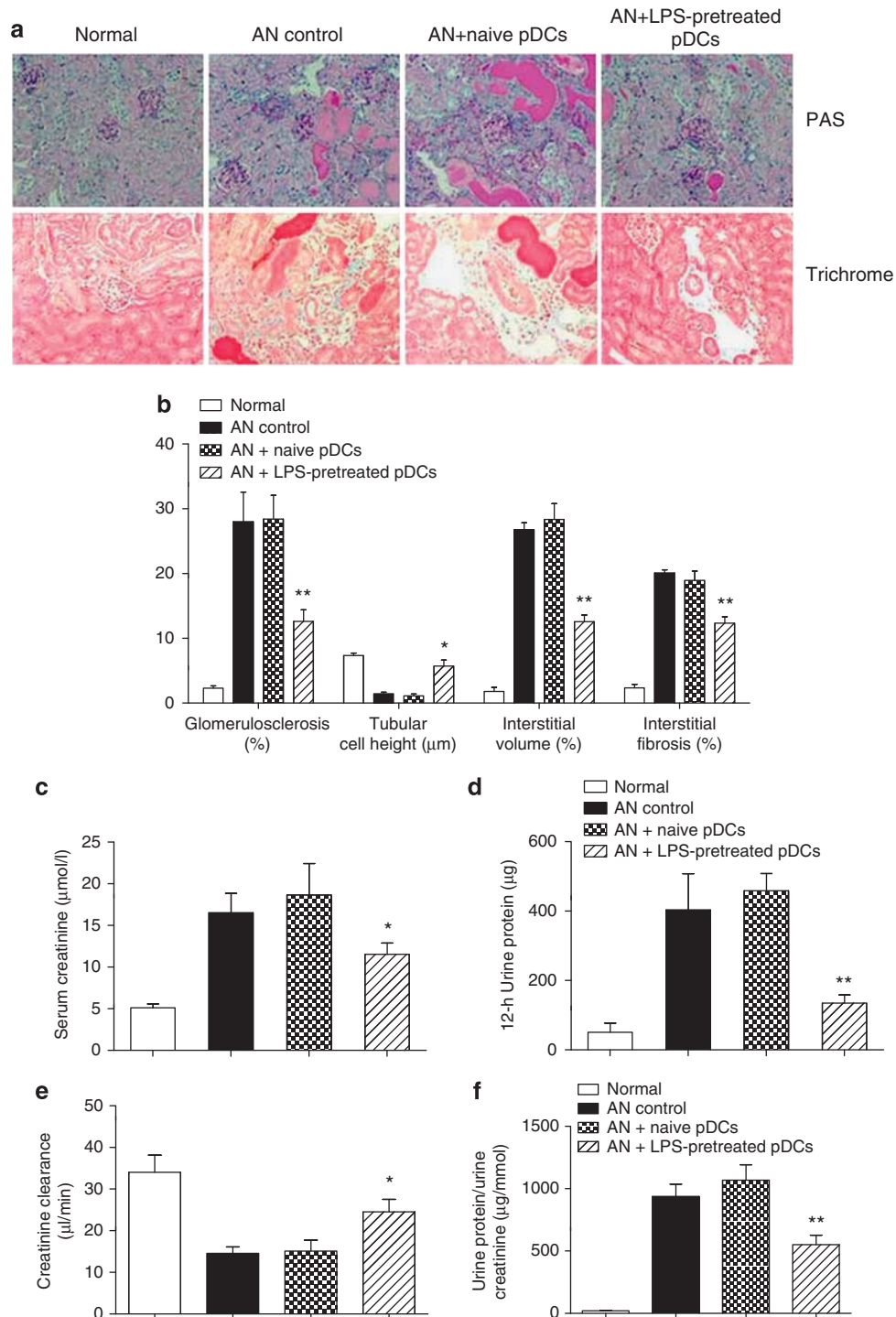
as shown by suppression of mRNA expression of pro-inflammatory cytokines tumor necrosis factor- $\alpha$ , IL-12, and inducible nitric oxide synthase, whereas naive pDCs had no effect. However, there were no differences in the expression of markers of protective macrophage (M2) mannose receptor, arginase, IL-10, and TGF- $\beta$  on M1 cocultured with naive pDCs versus LPS-pretreated pDCs (Figure 7a). Endogenous macrophages isolated from kidneys of each of the four groups were examined. Similar to the *in vitro* studies, the mRNA expression of tumor necrosis factor- $\alpha$ , IL-12, and inducible nitric oxide synthase of endogenous kidney macrophages was reduced significantly in AN mice receiving LPS-pretreated pDCs, but not naive pDCs, and there was no change in expression of M2 macrophages markers (Figure 7b). Together, these data suggested a further mechanism by which LPS-pretreated pDCs could prevent renal injury, that is, by deactivation of host macrophages.

## DISCUSSION

In this study, the effects of splenic pDCs were examined in murine AN. We demonstrated that splenic pDCs are able to reduce renal structural and functional injury and monocyte infiltration in AN. The mechanisms by which pDCs protected against renal injury appeared to involve their ability to convert CD4 + CD25 $^{-}$  T cells into CD4 + CD25 + Foxp3 + Tregs in KDLNs and kidney via increasing the expression of IDO and to suppress proinflammatory cytokine production of endogenous renal macrophages.

DCs largely reside in renal tubulointerstitium, forming an intricate immune sentinel network.<sup>24</sup> A quantitative evaluation of DC subsets has shown that both mDCs and

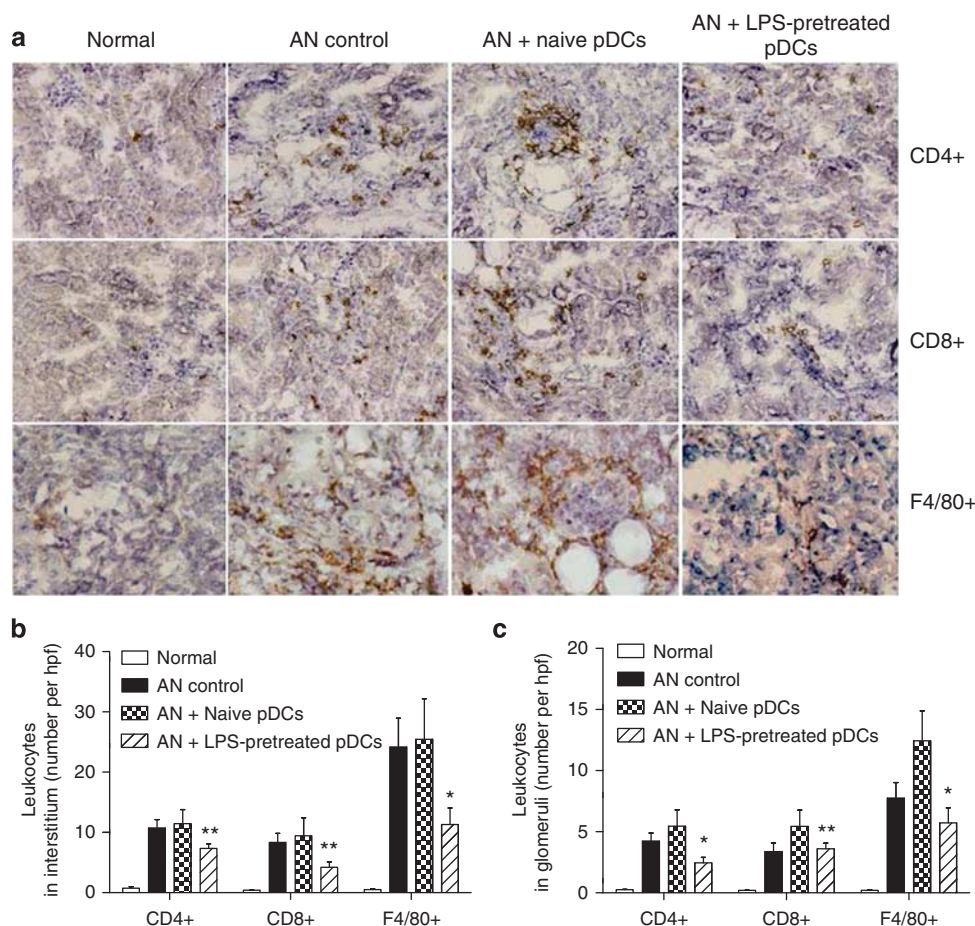
pDCs participate in inflammatory renal injury,<sup>25</sup> suggesting that both mDCs and pDCs play a role in tubulointerstitial injury under pathophysiological conditions. In addition, a significant number of pDCs have been found at sites of inflammation in lupus nephritis. Inhibition of interferon- $\alpha$  produced by pDCs with a specific TLR7 inhibitor IRS954 has been shown to ameliorate disease progression in lupus-prone mice.<sup>26</sup> Moreover, Coates *et al.*<sup>27</sup> demonstrated that Fms-like tyrosine kinase 3 ligand increased the number of CD11c + CD45RA + pDCs in glomeruli and interstitium of kidneys and that these pDCs were functionally immature and exhibited suppressive features. In general, pDCs play crucial roles in the development and resolution of renal inflammation. DCs are able to induce dynamic alterations in their function and phenotype to induce or suppress immune responses.<sup>28</sup> For example, depletion of renal DCs enhanced renal injury in nephrotoxic nephritis<sup>29,30</sup> and impaired recovery from ischemia/reperfusion injury,<sup>31</sup> indicating a protective role of DCs. However, depletion of renal activated DCs rapidly resolved established renal immunopathology in a mouse model of glomerular injury, suggesting a facilitatory role for DCs in the progression of renal diseases.<sup>32</sup> In the study of nephrotoxic nephritis, all DCs were depleted at an early point of disease and therefore the resident DCs were also removed.<sup>30</sup> It is possible that depletion of only these resident DCs was responsible for the worse renal injury. Thus, the effect of DCs in kidney may depend on the particular type of renal disease (for example, immune vs. nonimmune) or the functional state of renal DCs (for example, activated or resting; mature or immature). An alternative way to clarify the role of DCs in renal disease is the use of adoptive transfer.



**Figure 3 | Protective effect of transfused lipopolysaccharide (LPS)-pretreated plasmacytoid dendritic cells (pDCs) on renal structural and functional injury.** (a) Representative periodic acid-Schiff (PAS)- and trichrome-stained photomicrographs of kidneys 4 weeks after adriamycin from normal, adriamycin nephropathy (AN) control, AN + naive pDC, and AN + LPS-pretreated pDC groups are shown ( $\times 100$ ). (b) Kidney injury and fibrosis were assessed quantitatively on PAS- and trichrome-stained sections, respectively. (c-f) Serum creatinine, 12 h urinary protein, creatinine clearance, and ratio of urine protein to creatinine were assessed in normal, AN control, AN + naive pDC, and AN + LPS-pretreated pDC groups at 4 weeks. The data represent the mean  $\pm$  s.d. of each group. \*\* $P < 0.01$  and \* $P < 0.05$  vs. AN control and AN + naive pDCs.

We adoptively transferred unstimulated (naive) and pDCs stimulated with LPS into AN mice, and showed protective effects with stimulated but not naive pDCs.

pDCs modified by CpG have been shown to induce allogeneic T-cell hyporesponsiveness and prolong cardiac graft survival.<sup>10</sup> Furthermore, pDCs modified by LPS have also

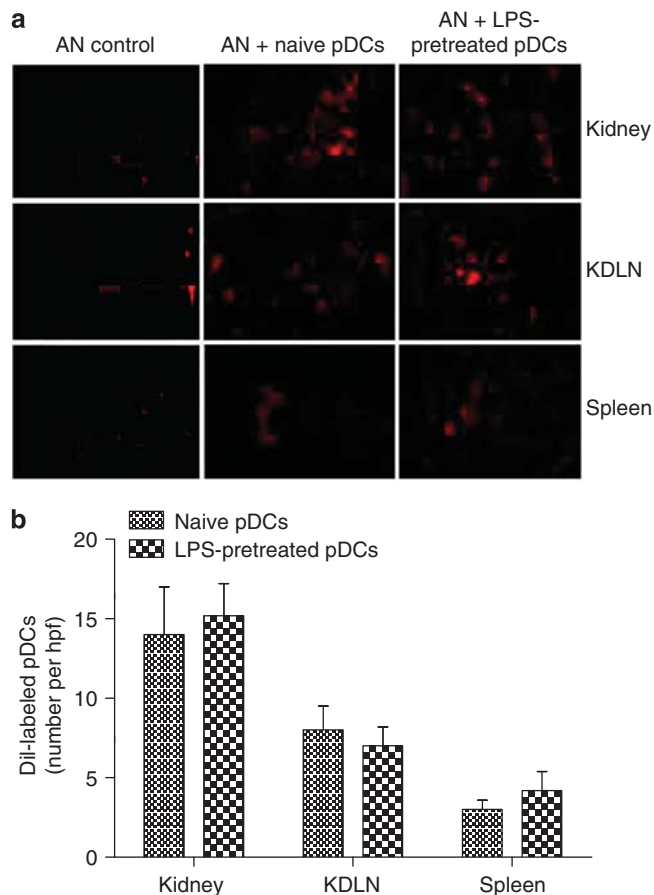


**Figure 4 | Quantitative analysis of renal cortical inflammatory cells.** (a) Cells expressing CD4, CD8, and F4/80 (brown) were stained on immunohistological sections from all four groups ( $\times 100$ ). (b, c) The number of F4/80+ macrophages, CD4+ T cells, and CD8+ T cells in (b) interstitium and (c) glomeruli were quantified by point counting. hpf, high-power field. Data represent the mean  $\pm$  s.d. of each group. \*\* $P < 0.01$  and \* $P < 0.05$  lipopolysaccharide (LPS)-pretreated plasmacytoid dendritic cells (pDCs) vs. AN control and AN + naive pDCs.

been shown to be capable of suppressing experimental autoimmune encephalomyelitis.<sup>12</sup> In this study, we examined the suppressive capacity of both LPS-pretreated and naive pDCs. pDCs pretreated with LPS showed high expression of B7-H1, IDO, and the anti-inflammatory cytokines IL-10 and TGF- $\beta$ . Both CpG- and LPS-pretreated pDCs expressed high levels of B7-H1. B7-H1 has been shown to down-regulate T-cell activation.<sup>33,34</sup> Increasing B7-H1 expression in pDCs has been shown to induce allogeneic T-cell hyporesponsiveness and prolong graft survival.<sup>10</sup> pDCs also express high levels of IDO after LPS or CpG stimulation. IDO is a component of the enzymatic pathway involved in the catabolism of tryptophan. Tryptophan depletion has been shown to suppress proliferation of activated T cells.<sup>35,36</sup> The role of IDO in regulatory function of pDCs has been addressed previously.<sup>37</sup> CpG oligodeoxynucleotide activation of pDCs significantly increased their expression of IDO and resulted in the generation of inducible Tregs from CD4+ CD25- T cells.<sup>38</sup> In this study, we found that LPS-modulated pDCs also expressed high levels of IDO.

It has been shown that pDC were able to suppress autoimmune encephalomyelitis following their exposure to LPS,<sup>12</sup> and to induce tolerance in experimental models of asthma, and in allogeneic hematopoietic stem cell, heart, and liver transplantation.<sup>10,39-41</sup> However, whether pDCs can be used to treat chronic kidney disease is unknown. This study, for first time, showed that LPS-pretreated pDCs can reduce glomerulosclerosis, tubulointerstitial injury, and interstitial fibrosis, and improve renal function in a model of chronic proteinuric renal disease. pDCs, both naive and pretreated with LPS, were able to reach the site of injury within kidneys, and to migrate to KDLNs. However, pDCs modified with LPS *ex vivo* into a regulatory phenotype, but not unstimulated pDCs, could ameliorate renal injury and reduce renal infiltration with inflammatory cells. Thus, it is their phenotype and not access to sites of inflammation that distinguishes LPS-pretreated pDCs from naive pDCs, and determines their protective effect.

In this study, LPS-pretreated pDCs showed inhibitory functions, yet the mechanism underlying pDC modulation by LPS into a regulatory phenotype is unknown. We found



**Figure 5 | *In vivo* tracking of transfused plasmacytoid dendritic cells (pDCs).** (a) Dil-labeled pDCs were seen in kidney, kidney-draining lymph nodes (KDLNs), and spleen at day 28 after adriamycin (ADR) administration. (b) Numbers of Dil-labeled naive or lipopolysaccharide (LPS)-pretreated pDCs were counted. Data represent the mean  $\pm$  s.d. per high-power field (hpf,  $\times 100$ ),  $N = 7$  per each group.

that some pDCs after LPS treatment had increased expression of the LPS receptor TLR4, consistent with its involvement in the effect of LPS on pDCs. This result is in line with that of others investigators who showed that TLR4 was significantly increased after LPS in murine liver and thymic pDCs.<sup>42,43</sup> However, in our study and similar to that of others, only 18% of pDCs expressed TLR4, suggesting that the effects of LPS might be mediated by other unknown pathways not involving TLR4. It has been reported that pDCs can be induced to express IDO under inflammatory conditions in humans and mice;<sup>44</sup> however, how LPS induces IDO is not clear.

This study explored several potential mechanisms by which adoptive transfer of LPS-pretreated pDCs might reduce renal injury. First, we examined an effect on macrophages. LPS-pretreated pDCs suppressed endogenous macrophages by reducing their expression of inducible nitric oxide synthase and inflammatory cytokines. Inducible nitric oxide synthase, tumor necrosis factor- $\alpha$ , and IL-12 from

macrophages have been demonstrated to be important mediators of renal inflammation and fibrosis in many types of chronic kidney disease.<sup>45–47</sup> Endogenous renal macrophages suppressed by LPS-pretreated pDCs secreted less proinflammatory cytokines that in turn could have reduced accumulation of T cells and macrophages. In this study we did not find any evidence that LPS-pretreated pDCs were able to convert M1 macrophages *in vitro* or effector macrophages *in vivo* to alternatively activated or protective macrophages (M2). Next, we examined an effect on Tregs. Interestingly, Treg numbers were significantly increased in KDLNs and kidneys after adoptive transfer of LPS-pretreated pDCs. Moreover, *in vitro*, LPS-pretreated pDCs were able to convert CD4 + CD25– T cells into Foxp3 + Tregs. When IDO was blocked, LPS-pretreated pDCs did not increase the number of Foxp3 + Tregs, suggesting that the effects of pDCs on T cells are IDO dependent. This result recalls previous studies in which human pDCs activated by human immunodeficiency virus induced Tregs through an IDO-dependent mechanism.<sup>48,49</sup>

In conclusion, we have demonstrated that LPS-pretreated pDCs can protect against renal functional and histological injury in mice with AN. The mechanisms underlying the protective effects of LPS-pretreated pDCs may involve their IDO-dependent ability to transform naive T cells into Tregs in kidney and lymph nodes and to suppress proinflammatory cytokine production of macrophages. The regulatory ability of pDCs may provide a novel and effective therapeutic approach for chronic kidney disease.

## MATERIALS AND METHODS

### Murine AN model

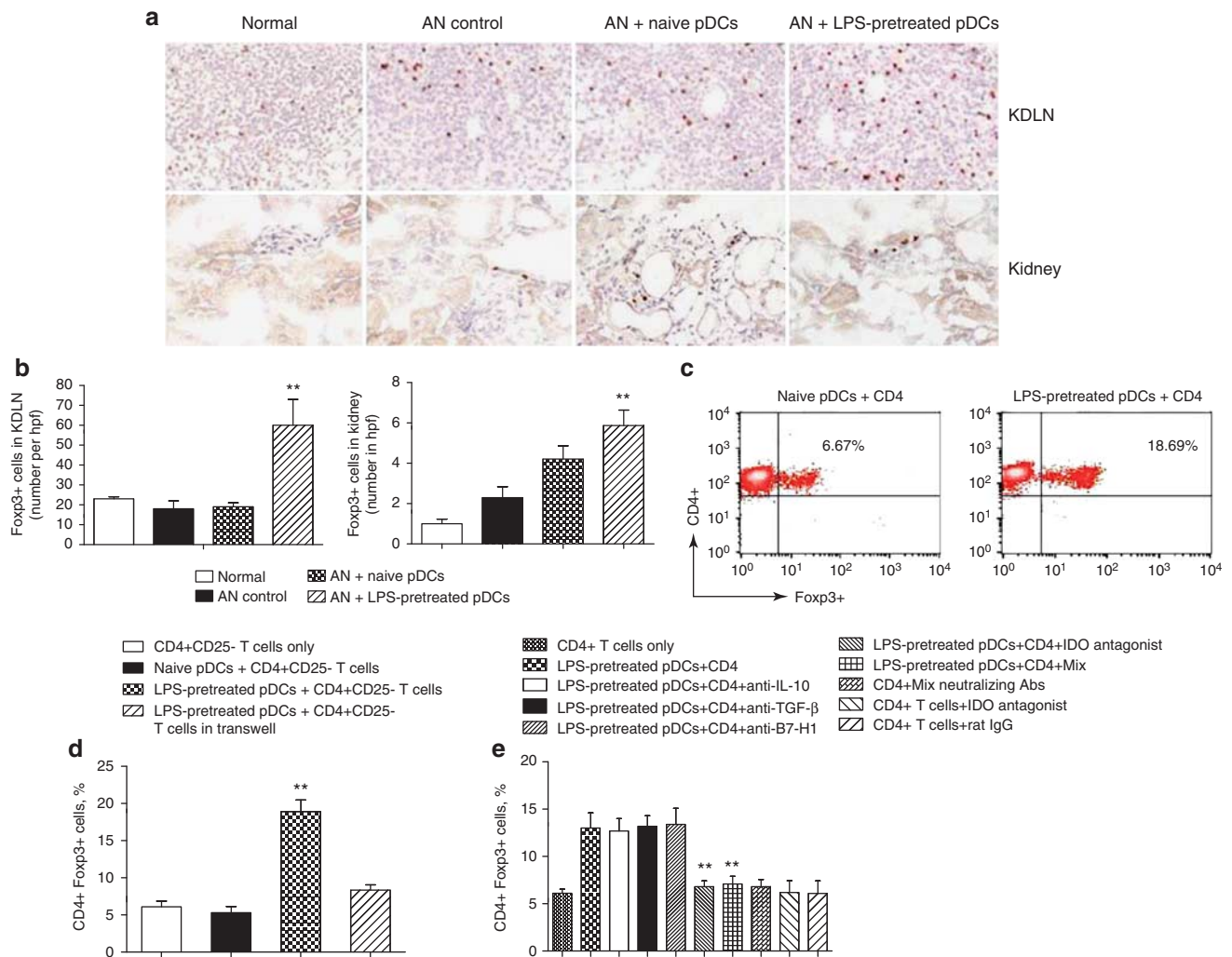
Male BALB/c mice, 6 to 8 weeks old, obtained from the Animal Resources Centre (Perth, Australia) were used in this study. The Animal Ethics Committee of Westmead Hospital approved all procedures. Adriamycin (9.8 mg/kg) was injected once via the tail vein of each non-anesthetized BALB/c mouse 5 days before pDCs were transfused into mice. Mice were divided into four groups ( $n = 7$  per group); normal, AN control, AN + naive pDC, and AN + LPS-pretreated pDCs. Mice were killed on day 28 after adriamycin administration. Kidney, KDLNs, and spleens were examined.

### pDC isolation and activation

pDCs were purified from spleen using anti-mPDCA-1 microbeads (from Miltenyi Biotec, Teterow, Germany). A total of 92.3% of separated cells were CD11c + B220 + (pDC marker), 0.4% CD11c + B220– (bone marrow DC marker); 0.07% CD49b + (natural killer cell marker); 0.2% CD19 + (B-cell marker); 2.4% CD11b + (macrophage marker); and 7.6% CD4 + (T-cell marker). pDCs were stimulated with LPS (0.5  $\mu$ g/ml, Sigma) for 24 h to become LPS-pretreated pDCs.

### Quantitative reverse transcription-PCR

RNA was isolated and reverse-transcribed with cDNA synthesis kit (Invitrogen, Carlsbad, CA), and real-time PCR using the SYBR (Invitrogen). The analysis method has been described previously.<sup>50</sup> The PCR primer sequences are presented in Table 1.



**Figure 6 | Induction of regulatory T cells (Tregs) *in vivo* and *in vitro*.** (a) Foxp3+ staining of kidney and kidney-draining lymph nodes (KDLNs) of all four groups ( $\times 200$ ). (b) Numbers of Foxp3+ cells in KDLN and kidney were assessed quantitatively.  $**P < 0.01$  vs. adriamycin nephropathy (AN) and AN + naive plasmacytoid dendritic cell (pDC) group. hpf, high-power field. (c, d) Naive pDCs and lipopolysaccharide (LPS)-pretreated pDCs were cocultured with CD4 + CD25- T cells (CD4) *in vitro*. The percentage of Foxp3+ cells among total CD4+ T cells was measured by fluorescence-activated cell sorting (FACS). Transwell was used to examine dependency on cell contact. (e) Neutralizing antibodies (Abs) and isotype antibody against interleukin-10 (IL-10), transforming growth factor- $\beta$  (TGF- $\beta$ ) or B7-H1, indoleamine 2,3-dioxygenase (IDO) blockade reagent 1-methyl-D-tryptophan (D-1MT), or a mixture of all four inhibitors (mix) were used to block the effects of LPS-pretreated pDCs in inducing Foxp3 expression on CD4 + CD25- T cells in coculture (CD4). Data represent the mean  $\pm$  s.d. of three separate experiments.  $**P < 0.01$  vs. anti-IL-10, anti-TGF- $\beta$  and anti-B7-H1 groups.

### Enzyme-linked immunosorbent assay (ELISA)

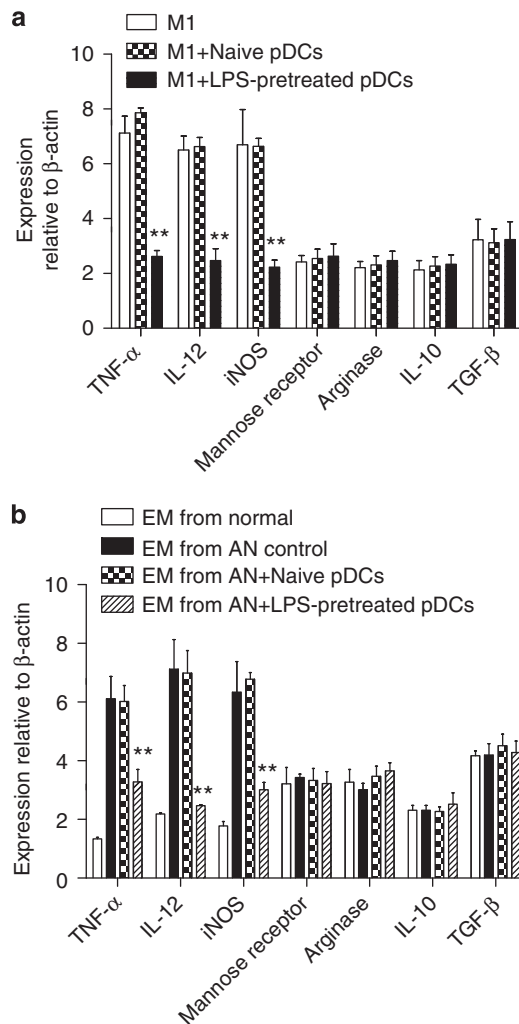
IL-12, TGF- $\beta$ , and IL-10 protein levels were measured in culture supernatants using IL-12, TGF- $\beta$ , and IL-10 ELISA kits (R&D Systems, Minneapolis, MN), and for IDO measurement, the cellular extracts were obtained by lysis of  $1 \times 10^5$  pDCs in lysis buffer and assessed with IDO ELISA kit (antibodies-online GmbH, Atlanta, GA) according to the manufacturer's instructions.

### Flow cytometry analysis

Naive pDCs were suspended in fluorescence-activated cell sorter wash buffer (phosphate-buffered saline) and CD32/16 Fc block (eBiosciences, San Diego, CA). To analyze purity of naive pDCs, cells were stained with fluorescein isothiocyanate-conjugated anti-mouse B220 (Miltenyi Biotec, Bergisch Gladbach, Germany), CD49b

(Biolegend, San Diego, CA), CD4 (BD Biosciences, San Jose, CA), CD8 (eBiosciences), and phycoerythrin-conjugated anti-mouse CD11c (eBiosciences) and CD11b (eBiosciences). To analyze pDC surface antigen expression, phycoerythrin-conjugated anti-mouse B7-H1, B7-H2, B7-H3, and B7-H4 (all from eBiosciences) were used. For intracellular staining of *Foxp3*, T cells were fixed and stained with phycoerythrin-Cy5-conjugated anti-mouse *Foxp3* (eBiosciences). Armenian hamster IgG was used as control antibody for CD11c; rat IgG2a for the antibodies to CD19, B7-H1, B7-H2, and *Foxp3*; and rat IgG2b for antibodies to CD11b, B7-H3, and B7-H4. Flow cytometry was performed using a fluorescence-activated cell sorter Calibur cytometer (BD Biosciences). Percentage of positive cells was analyzed using Cellquest software and FlowJo (Tree Star, Ashland, OR) in comparison with fluorescence-labeled isotype controls.





**Figure 7 | Lipopolysaccharide (LPS)-pretreated plasmacytoid dendritic cell (pDC) suppression of effector macrophages (M1) *in vitro* and *in vivo*.** (a) M1 were cocultured with pDCs *in vitro* for 24 h. The mRNA expression of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-12 (IL-12), inducible nitric oxide synthase (iNOS), mannose receptor, arginase, IL-10, and transforming growth factor- $\beta$  (TGF- $\beta$ ) of M1 was examined by real-time PCR.  $**P < 0.01$  vs. M1 and M1 + naive pDCs. (b) Endogenous macrophages (EMs) were isolated from kidney of each of the four groups. The mRNA expression of TNF- $\alpha$ , IL-12, iNOS, mannose receptor, arginase, IL-10, and TGF- $\beta$  of macrophages was examined by real-time PCR. AN, adriamycin nephropathy.  $**P < 0.01$  vs. M1 and M1 + naive pDCs.

**Coculture experiments**

For Foxp3 + Treg induction experiments, CD4 + CD25- T cells were cocultured with freshly isolated naive pDCs or LPS-pretreated pDCs for 7 days. For transwell experiments, CD4 + CD25- T cells and LPS-pretreated pDCs were cocultured in 24-well plates and placed separately in transwell chambers (0.4  $\mu$ m, Millipore, Kilsy, VIC, Australia). For neutralizing antibody blocking, CD4 + CD25- T cells and LPS-pretreated pDCs were cocultured in 24-well plates in the presence of IL-10-, TGF- $\beta$ -, and B7-H1-neutralizing antibodies (10  $\mu$ g/ml each) for 7 days. D-1MT (Sigma-Aldrich, St Louis, MO) was prepared as a 20 mmol/l stock solution in 0.1 M NaOH, adjusted to pH 7.4, and stored at -20  $^{\circ}$ C protected from light. For *in vitro*

**Table 1 | Real-time PCR primers**

Gene	Primer sequence (5'-3')	Product
IL-10 (F)	CCAGTACAGCCGGGAAGACA	121
IL-10 (R)	CAGCTGGTCCTTTGTTTGAAGA	
TGF- $\beta$ (F)	TTAGGAAGGACCTGGGTTGGA	121
TGF- $\beta$ (R)	AGGGCAAGGACCTTGCTGTA	
TNF- $\alpha$ (F)	GCTGAGCTCAAACCTGGTA	118
TNF- $\alpha$ (R)	CGGACTCCGCAAAGTCTAAG	
iNOS (F)	CACCTTGGAGTTCACCCAGT	170
iNOS (R)	ACCACTCGTACTGGGATGC	
IDO (F)	AAGGGCTTCTTCTCGTCTC	200
IDO (R)	AAAAACGTGTCTGGGTCCAC	
IL-12 (F)	ACGGCCAGAGAAAACTGAA	218
IL-12 (R)	CTACCAAGGCACAGGGTCAT	
IFN- $\alpha$ (F)	CTACTGGCCAACCTGCTCTC	258
IFN- $\alpha$ (R)	TTCCATGCAGCAGATGAGTC	

Abbreviations: F, forward; IDO, indoleamine 2,3-dioxygenase; IFN- $\alpha$ , interferon- $\alpha$ ; IL-10, interleukin-10; iNOS, inducible nitric oxide synthase; R, reverse; TGF- $\beta$ , transforming growth factor- $\beta$ ; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ .

use, D-1MT was added in a final concentration of 100  $\mu$ M into coculture medium. After incubation, CD4 + T cells were stained with Foxp3 antibody and analyzed by fluorescence-activated cell sorting. Splenic macrophages were separated by CD11b microbeads (Mitenyi Biotech, Bergisch Gladbach, Germany) and were grown on the bottom of a 24-well plate with LPS for 2 h to become effector macrophages (M1). pDCs were added onto the same plate and cocultured with M1 for 24 h. After incubation, expression of markers of effector (M1) and protective (M2) macrophages were examined.

**pDC DiI labeling and adoptive transfer to BALB/c mice**

Naive pDC and LPS-pretreated pDCs were labeled with DiI (Invitrogen).<sup>51</sup> One million cells were transferred into AN-treated mice by a single tail vein injection at day 5 after adriamycin administration.

**Renal function**

All urine and blood samples were prepared and examined by the Institute of Clinical Pathology and Medical Research (Westmead Hospital, Sydney, Australia).

**Histology**

Coronal sections of renal tissue were immersion-fixed in 10% neutral buffered formalin and embedded in paraffin. Sections 5  $\mu$ m thick were stained with periodic acid-Schiff or trichrome. To quantitate tubular atrophy, the tubule cell height of individual cortical tubules was measured using line morphometry (magnification  $\times$  200) by computer image analysis software (ImageJ, Rockville, MD).<sup>52</sup> The tubular diameter was defined as the length of a straight line that passed through the center of a symmetrically sectioned tubule and joining two points on the tubular circumference. A total of 50 randomly selected cortical tubules in 10 nonoverlapping fields (magnification  $\times$  200) were measured, and the mean cross-sectional tubule cell height was determined for each section. The cortical interstitial volume included the tubular basement membrane and peritubular capillaries. To quantitate this area, cortical fields (magnification  $\times$  200) were viewed on a video screen, and the area of interstitial space was determined with image analysis software and was expressed as a percentage of the total area of the field. The mean percentage area of five non-overlapping cortical fields was calculated for each section. Interstitial fibrosis was assessed and quantified on

trichrome-stained sections by point counting using ImageJ software in each of 10 non-overlapping randomly selected cortical fields. Points falling within blue areas (fibrosis) were considered as positive. Scores derived from 10 fields per kidney section (3 sections/per mouse) were collected for the analyses.

### Immunohistochemistry

For immunohistochemical staining of macrophages, CD4<sup>+</sup>, and CD8<sup>+</sup> cells, rat anti-mouse F4/80, CD4, and CD8 antibodies (BD Pharmingen, Franklin Lakes, NJ) were used as the primary antibodies. Biotinylated peroxidase (ZYMED, South San Francisco, CA) and Streptavidin (ZYMED) were used for the secondary antibodies. Kidney sections were placed in optimal cutting temperature compound (Sakura Fintek, Torrance, CA). Sections of 5 µm were cut, dried overnight, and fixed in cold acetone for 8 min. Endogenous peroxidase activity was blocked by 0.3% (v/v) H<sub>2</sub>O<sub>2</sub> solution for 15 min when incubating the slides. Biotin Blocking System (Dako, Carpinteria, CA) was used to block endogenous avidin-binding activity. Normal rat immunoglobulin was used for control sections. Sections were incubated with biotinylated polyclonal rabbit anti-rat immunoglobulin (Dako Corporation, Glostrup, Denmark) or RTU Vectastain Elite ABC Peroxidase Kit (Vector Laboratories, Burlingame, CA), and 3,3'-diaminobenzidine substrate chromogen solution (Dako) was applied and then washed. Slides were counterstained with hematoxylin (Sigma-Aldrich, Steinheim, Germany). For assessment of interstitial inflammation, positively stained cells located in the interstitial area were counted from more than 20 random cortical fields (magnification × 200) in each section, and the numbers averaged for each section.

### Statistics

Renal functional data were log-transformed before analysis to stabilize the variance. Statistical tests included unpaired two-tailed Student's test using one-way analysis of variance with Tukey's multiple comparison test. Statistical analyses were done using Prism (Version 4, Graphpad, La Jolla, CA). Results are expressed as the mean ± s.d. A *P*-value of <0.05 was considered statistically significant.

### DISCLOSURE

All the authors declared no competing interests.

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