Chapter 6

Human renal fibroblasts generate Dendritic cells with a unique regulatory profile.

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Abstract
Fibroblasts reside within the renal interstitium in close proximity to neighbouring Dendritic cells (DCs). It is likely that these cells play a central role in the maintenance and function of resident and infiltrating renal DCs, though studies to confirm this have been lacking. We investigated whether renal fibroblasts influence human DC generation and function. We found that co-culture with renal fibroblasts led to the generation of monocyte derived dendritic cells (Fibro-DCs) with significantly reduced CD80, CD83 and CD86, but elevated B7H1 and B7DC expression. In addition these Fibro-DCs displayed a reduced capacity to produce IL-12p40 and IL-12p70 but maintained normal levels of IL-23 and IL-27. Furthermore IL-10 production was elevated, which together resulted in a regulatory DC population with a profoundly reduced capacity to stimulate allogenic T cell proliferation and IFNγ production, while preserving IL-17A. Supernatant transfer experiments suggested that a soluble mediator from the fibroblasts was sufficient to inhibit the immunogenic capability of DCs. Further experiments demonstrated that IL-6 was at least partially responsible for the modulating effect of renal fibroblasts on DC generation and subsequent function. In summary, renal fibroblasts may play a crucial decisive role in regulating local DC immune responses in vivo. Better understanding into this cell population and their mechanisms of action may have therapeutic relevance in many immune-driven renal diseases.

Introduction
Dendritic cells (DC) represent a heterogeneous family of professional antigen presenting cells (APCs) that have many functions in both the initiation and maintenance of immunity and immunological tolerance[1]. Renal dendritic cells (rDCs) are a major constituent of the mononuclear phagocytic system within normal kidneys[2-4] , but the generation and function of the rDC network is not yet fully understood[5,6].
Tissue resident DCs are thought to be immature resting sentinels, preserving the immune homeostasis of their environment. During inflammation they defend the host tissue by shaping a local immune response to avoid destructive excessive inflammatory responses and to in turn promote peripheral tolerance[7,8]. Some of the strongest evidence in supporting the hypothesis that individual tissues have the ability to educate their own APCs[9] , are several studies focusing on stromal or mesenchymal stem cells[10-12]. DC differentiation in the presence of tissue-dependent microenvironment leads to a more regulatory cell population with lower expression of co-stimulatory molecules and poor induction of T cell proliferation. This process is described for several organs[8,13] including the bone marrow niche, spleen, lung[14], thymus and intestine[15]. More recently the presence

of kidney derived MSC like cells has been identified in mouse[16] although their exact location in vivo has yet to be fully discerned. It has been suggested that renal fibroblasts may be a stromal lineage of kidney MSCs, thereby warranting further investigation into their immunological contribution.

Renal fibroblasts have been shown to be in close proximity to rDCs in mouse kidneys[17]. Due to their prime location it is conceivable that interstitial renal fibroblasts are key in orchestrating a fully integrated immune response and may play a crucial role in educating local and infiltrating DCs. The immunomodulatory properties of human fibroblasts are still unclear however gingival fibroblasts have been shown to suppress proliferation of T cells[18], and inhibit monocyte derived DC generation[19]. Additionally fibroblasts have been demonstrated to play a direct role in guiding the development of regulatory DCs in spleen[20], while dermal fibroblasts have been shown to switch monocyte differentiation to macrophages rather than DCs[11]. Several factors have been implicated in the modulatory effect of stromal and fibroblasts on DCs including VEGF[19], IL-6[11], PGE2[21] and C3[22] however full investigation into the phenotype and function of these subsequent regulatory DCs has been limited.

Cytokines are key mediators involved in directing and maintaining a specific immune response as well as controlling inflammatory responses. One cytokine family which is gaining increasing attention, with the ability to modulate T cell activity in both a pro and anti-inflammatory way, is the IL-12 family[23]. The members, including IL-12, IL-23, IL-27 and IL-35, are heterodimeric cytokines, composed of an alpha and beta chain, which they share among each other. The subunit IL-12p40 can pair with IL-12p35 or IL-23p19 to form IL-12 or IL-23 respectively and Ebi3 can match with either IL-27p28 or IL-12p35 to form IL-27 or IL-35 respectively. IL-12 and IL-23 are mainly immunogenic cytokines, whereas IL-27 and IL-35 are more regulatory.

In this study we demonstrated that renal fibroblasts, partially via IL-6 secretion, led to the generation of a DC with decreased expression of immunogenic markers, reduced allostimulatory capabilities, and a distinct cytokine profile, with decreased levels of IL-12 but not IL-23, IL-27 or IL-10. Further investigation into the mechanisms involved may be important in fully understanding immune mediated renal diseases.

Material and Methods

Cell Culture and Reagents

DCs were generated as previously described[24]. On day 6, DCs were stimulated with 100ng/ml recombinant human IFN-γ (Peprotech, Germany) and 200ng/ml LPS (E.Coli EH100 Enzo, Belgium), CD40L or a combination of LPS+CD40L. CD40L activation was performed with irradiated (70Gy) CD40L-transfected L cells at an L cell: DC ratio of 1:5. Non-transfected L cells served as a control. DCs were plated at $1.0x10^6$/well

for 24 or 48hrs. Afterwards cells and supernatants were analysed as described.

Fibro-DC generation

The established human renal fibroblast cell line TK173, which has many characteristics in common with primary renal fibroblasts[25,26], characterised by the expression of CD73 and PDGRFβ, was cultured in RPMI 1640 with 10% FCS. The cells were harvested, irradiated (70Gy) and allowed to adhere overnight. The cultures were then washed to ensure only viable adherent cells remained, prior to co-culture with monocytes at a ratio of 1:7.5 at day 0. Day 6 DCs, co-cultured with fibroblasts, were carefully flushed from adherent fibroblasts and used similarly to Ctrl-DCs.

Flow Cytometry

Cells were harvested, washed in buffer (1% BSA, 0.5% heat-inactivated NHS and 0.02% NaN_3 in PBS) and stained for 30 minutes at 4ºC. Antibodies included DC-SIGN (R&D Systems, UK), Mannose-Receptor (clone D547), CD80, B7H1, B7DC (BioLegend, the Netherlands), CD14, CD86, CD1a-FITC, CD83-APC and HLA-DR-APC (BD biosciences, the Netherlands). Non-conjugated antibodies were visualised by PE-conjugated GaM-IgG1 (DAKO, Belgium). Cells were assessed for fluorescence intensity by flow cytometry (FACS caliber). Data was analysed by FlowJo Software (Treestar, USA).

mRNA isolation, cDNA synthesis and RT-PCR

DCs were harvested after 6hrs stimulation and mRNA was isolated using an RNeasy kit (Qiagen, Germany). Reverse transcriptase system kit (Promega, The Netherlands) was used to synthesise cDNA according to the manufacture's instruction. cDNA was amplified by RT-PCR using primers for the following genes: *IL12B, IL12A, IL23A, IL27B, IL27A* and *IL10* (Table I) and SYBR Green qPCR master mix (Bio-Rad, the Netherlands). *GAPDH* was used as the endogenous reference gene. Data analysis was performed using Bio-Rad CFX Manager Software.

Table I: Real Time PCR Oligonucleotide sequences

Gene	NCBI ID	Protein ID	Forward Sequence (5`-3`)	Reverse Sequence (5`-3`)
IL12B	3593	IL-12p40	CAGCAGCTTCTTCATCAGGG	GAGTACTCCAGGTGTCAGG
IL12A	3592	IL-12p35	CCAGAGTCCCGGGAAAGTC	ACCAGGGTAGCCACAAGG
IL27B	10148	Ebi3	TGGATCCGTTACAAGCGTC	AGTTCCCCGTAGTCTGTG
IL27A	246778	IL-27p28	GAGGGAGTTCACAGTCAGC	GCAGGAGGTACAGGTTCAC
IL23A	51561	IL-23p19	CCAAGGACTCAGGGACAAC	CTGAGGCTTGGAATCTGCTG
IL10	3586	IL-10	GCGCTGTCATCGATTTCTTCC	GTAGATGCCTTTCTCTTGGAGCTTA
GAPDH	2597	GAPDH	TTCCAGGAGCGAGATCCCT	CACCCATGACGAACATGGG

Cytokine production

Cell culture supernatants were harvested and stored until analysis. Levels of IL-12p40, IL-12p70 (BioLegend), IL-23, IL-27, IL-6 (eBioscience, Austria) and IL-10 (Sanquin, the Netherlands) were determined by ELISA according to the manufacturer's instructions. Supernatant of the DC:T cell co-culture was tested for the presence of IFNγ and IL-17A (eBioscience).

DC:T cell co culture

Allogeneic $CD4^+$ T cells were isolated from buffy coats by negative selection using the MACS $CD4^+$ T Cell Isolation Kit II (Miltenyi Biotec). DCs from all conditions were harvested after 24hrs, washed, and plated in

96-well round bottom plates at a starting ratio of at 1:20 with $0.1x10^6$ T cells. Cells were cultured for 5 days and supernatant was harvested to measure IFNγ and IL-17A production by means of ELISA. Proliferation was assessed by the addition of ^{3}[H]-thymidine (0,5 uCi/well) for the last 18hrs.

Microscopy

For light-microscopy, paraffin-embedded tissue sections were deparaffinised and rehydrated followed by heat-antigen retrieval using TRIS-EDTA-buffer. Slides were then washed in PBS and endogenous peroxidases were blocked with 0,4% H_2O_2 followed by 1% BSA/1% NHS in PBS for 30 minutes. Slides were incubated with primary antibodies in 1% BSA/1% NHS in PBS for 30 minutes, using mouse anti human MR (R&D) or rabbit anti human PDGF-Receptor β (Abcam, UK), followed by detection using the respective mouse/rabbit EnVision+ System-HRP (DAKO) for 30 minutes. Slides were counter-stained using Mayer's hemalum solution. Cover slips were mounted with Entellan (Merck, Germany).

For immunofluorescence staining, frozen 4 µm sections were fixed in cold acetone and blocked as described for paraffin sections. Slides were then incubated with anti-MR (D547 IgG1) and anti-PDGFRβ, overnight at RT, and incubated on the next day with GaM-IgG1-Alexa568 (Molecular Probes, Europe BV, the Netherlands) and GaR-HRP (DAKO) for 1hr at RT. HRP conjugated antibodies were visualized by incubation with Tyramide-fluorescein isothiocyanate in tyramide buffer (NEN™ Life Science Products, Boston, USA). Nuclei were counterstained with Hoechst. Sections were mounted with DABCO glycerol.

Statistical analysis

Statistical analysis was performed with Graph Pad Prism (Graph Pad, CA) using a one-tailed t-test. P-values ≤ 0.05 were considered statistically significant.

Results

Renal DCs and fibroblasts are in close proximity to one another in the renal interstitial network.

We have previously demonstrated that myeloid DCs form an extensive network throughout the renal interstitium in human kidneys[3]. We assessed the expression of the Mannose Receptor (MR) as a marker of resident rDCs and observed an abundant expression of MR staining in the cortical interstitial region of normal human kidney. In contrast to murine kidney[27], we found little expression of MR present within glomeruli (Fig.1A). Fibroblasts, visualised by the expression of PDGFRβ, were also largely abundant in the interstitial network surrounding the tubuli. Distinct from MR staining, PDGFRβ was also highly expressed in glomeruli (Fig.1B). When performing double staining, we observed no double positive cells but did observe, in the tubulointerstitial area, that fibroblasts and rDCs appeared to be in close contact, implying that fibroblasts might locally affect the function of rDCs (Fig.1C).

Fibro-DCs have enhanced expression of the immunoregulatory markers B7H1 and B7DC compared to DCs.

To investigate the effect of renal fibroblasts on DC development and function we

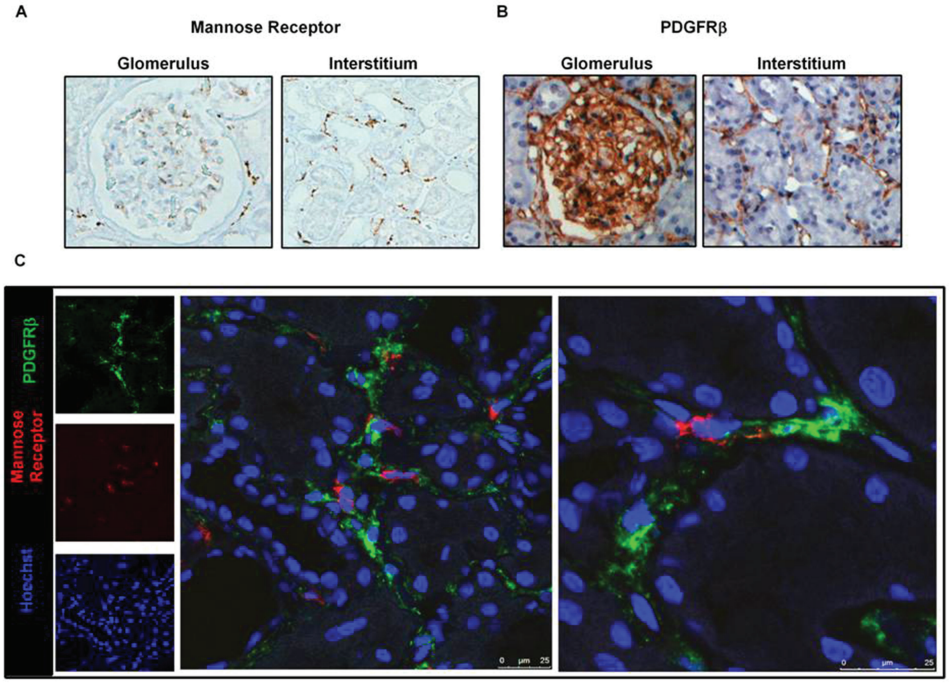

Figure 1: Dendritic cells and fibroblasts are both highly abundant within the renal interstitium and are often in close contact with one another. Paraffin sections of normal human kidney were stained for (A) Mannose Receptor or (B) PDGFRβ. (C) Immunofluorescent double stainings were performed on cryosections (4μm) of normal human kidneys for Mannose receptor (red), representing the DC network and PDGFRβ (green), representing the renal fibroblast network. Nuclei were stained with Hoechst (blue). Non-conjugated mouse and rabbit isotype antibodies were used to determine the level of non-specific background staining. Pictures are representative for at least five different donors.

generated DCs in the absence or presence of the well-established human renal fibroblast cell line, TK173[25]. Both Ctrl-DC and Fibro-DCs were morphologically similar on day 6 with the exception that the Fibro-DCs were often seen tethered to the co-cultured fibroblasts (Fig. 2A). Flow cytometric analysis of immature cells revealed that Ctrl-DC and Fibro-DCs expressed characteristic markers of DCs including low CD14 with high DC-SIGN and MR. However, CD1a expression was significantly reduced on Fibro-DCs compared to Ctrl-DC (Fig.2B,C). Further analysis showed that Fibro-DCs tended to express higher levels of CD80 and CD86 but this was not found to be significant. In contrast both B7H1 and B7DC were significantly upregulated on Fibro-DCs (Fig.2D,E). Next to a direct co-culture, we generated DCs in the presence of cell free fibroblast supernatant (DC+Fibro sup). On these cells, the reduced CD1a expression and elevated B7H1 and B7DC could not be demonstrated (Fig.2C,E), indicating that cell contact with renal fibroblasts is required for these particular phenotypical changes.

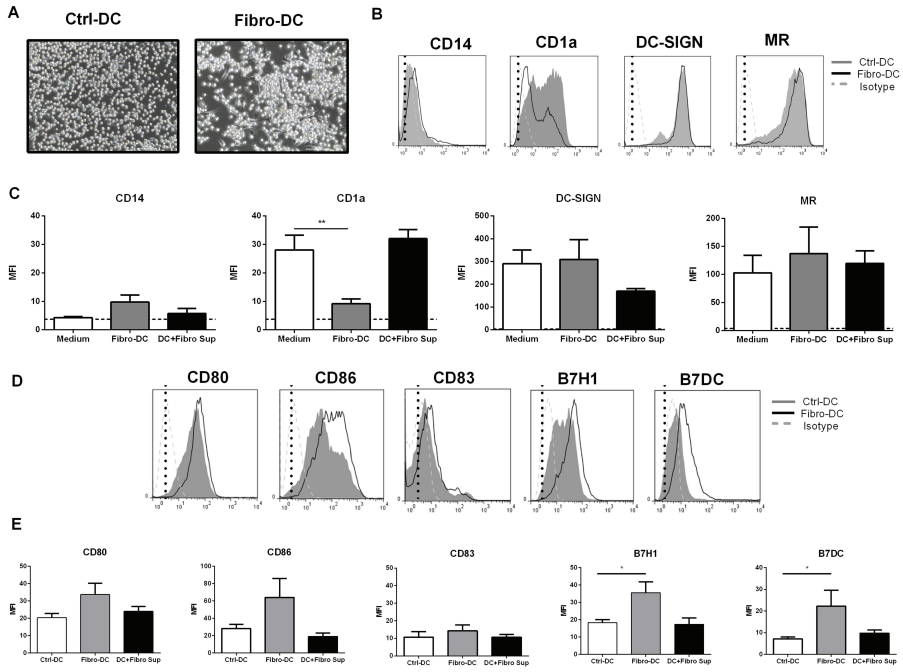

Figure 2: Fibro-DCs display reduced levels of CD1a but express typical markers of Ctrl-DC including DC-SIGN and Mannose Receptor. (A) DCs were generated as previously described and cultured for 6 days in the presence of IL-4 and GM-CSF to obtain Ctrl-DC. Fibro-DCs were generated by the addition of irradiated renal fibroblasts to the moDC cultures at day 0. The expression of monocytic and dendritic cell markers (B) were analysed on unstimulated cells; Ctrl-DC (filled histograms) and Fibro-DC (solid black line). Dashed lines represent isotype control mAb staining. Results are representative of 5-10 independent experiments. The expression of (C) CD14, CD1a, DC-SIGN and Mannose Receptor (MR) was analysed using flow cytometry. DC+Fibro sup cells were generated by addition of cell free fibroblast supernatant at day 0. Dashed line indicates the mean MFI of isotype controls used. Results shown are the mean ± SD of 5-10 independent experiments performed. The expression of co-stimulatory and inhibitory molecules (D) were analysed on unstimulated cells; Ctrl-DC (filled histograms) and Fibro-DC (solid black line). APC- and FITC-conjugated isotype antibodies were used to determine the level of background staining for directly labelled antibodies. Dashed lines represent isotype control mAb staining. The expression of (E) CD80, CD86, CD83, B7H1 and B7DC was analysed using flow cytometry. Dashed line indicates the mean MFI of isotype controls used. Results shown are the mean ± SD of 5-10 independent experiments performed.

Upon activation, Fibro-DCs display diminished levels of CD80 and CD86, yet enhanced levels of B7H1 and B7DC.

To further explore the phenotype of Fibro-DCs, cells were harvested and stimulated with IFNγ+LPS or CD40L. Both Ctrl-DCs and Fibro-DCs developed characteristic clusters of activated DCs with many cells also becoming more

adherent to plastic (Fig.3A). This was particularly evident for Fibro-DCs stimulated with IFNγ+LPS.

Upon activation with IFNγ+LPS both cell types increased expression of CD80, CD83, CD86, B7H1 and B7DC (Fig.3B), compared to non-activated cells (Fig.2D). Ctrl-DCs expressed high levels of CD80, CD86 and CD83, whereas mature Fibro-DCs expressed significantly lower levels (Fig.3C). This was in contrast to B7H1 and B7DC, where expression was higher on Fibro-DCs (Fig.3C). DCs generated in the presence of fibroblast supernatant showed reduced levels of CD80 and CD86, but did not demonstrate increased levels of B7H1 and B7DC (Fig.3C). This suggests that cell contact with renal fibroblasts is necessary to actively upregulate immunoregulatory markers.

The differential regulation of CD86 and B7H1 became more evident when calculating the stimulation index of individual experiments, where the more than 10 fold induction of CD86 expression on DCs, was significantly reduced on Fibro-DC (Fig.3D). As a consequence, the ratio of B7H1:CD86 showed a more than 2 fold increase on Fibro-DCs (Fig.3E).

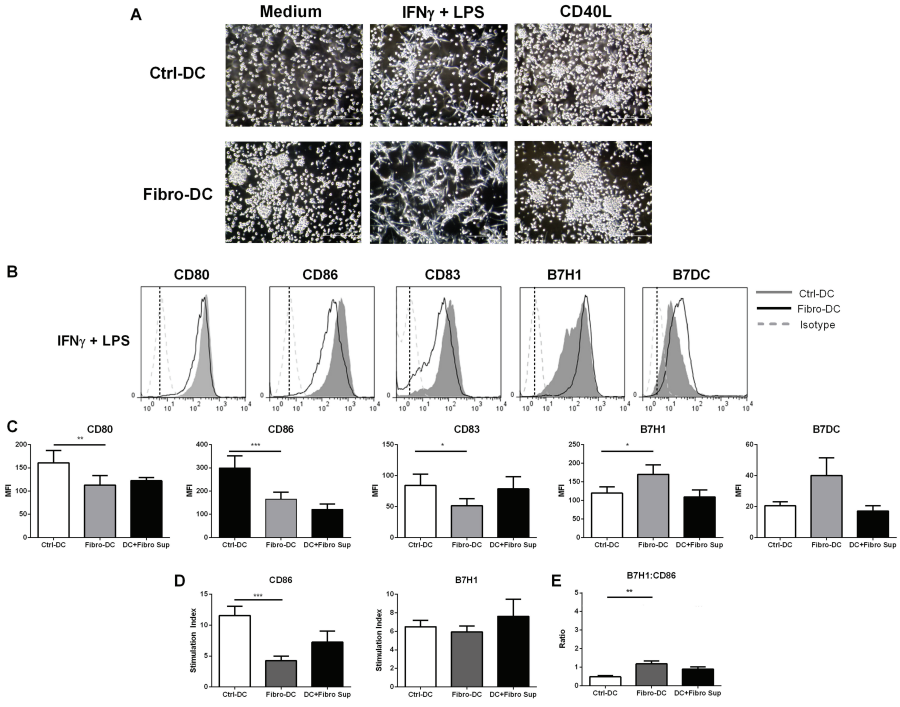

Figure 3: Fibro-DCs display typical phenotypical characteristics of regulatory DCs with reduced CD80, CD83 and CD86 but increased levels of B7DC and B7H1 upon stimulation. (A) Cells were harvested on day 6, replated and activated overnight with IFNγ+LPS or CD40L. The expression of (B) CD80, CD86, CD83, B7H1 and B7DC on Ctrl-DC (filled histograms) and Fibro-DC (solid black line) was analysed using flow cytometry. APC- and FITC-conjugated isotype antibodies were used to determine the level of background staining for directly labelled antibodies.

Dashed lines represent isotype control mAb staining. Results shown are from a representative experiment of 5-10 independent experiments performed. Data shown in (C) is the mean ± SD of those 5-10 experiments. The stimulation index was obtained by comparing expression levels of (D) CD86 and B7H1 upon stimulation with IFNγ+LPS compared to unstimulated cells. (E) The ratio of B7H1:CD86 was determined in IFNγ+LPS stimulated cells. Data shown in is the mean ± SD of 5-10 experiments.

Fibro-DCs display a reduced allostimulatory capacity.

A key hallmark of DCs is their unique capacity to induce T cell proliferation and cytokine production, so we assessed whether the reduced expression of CD80 and CD86 had an affect on T cell proliferation. Ctrl-DC, Fibro-DC or DC+Fibro Sup cells were co-cultured with allogenic $CD4^+$ T lymphocytes for 5 days, after which the T cells were analysed for proliferation and cytokine production (Fig.4). Ctrl-DCs were potent inducers of T cell proliferation while both Fibro-DCs and DC+Fibro sup conditions had a significantly reduced allostimulatory capacity (Fig.4A). Ligation of CD40 on Ctrl-DC prior to co-culture with T cells led to greater proliferation which was again reduced in Fibro-DC and DC+Fibro sup conditions (Fig.4B). A similarly reduced T cell stimulatory capacity was also observed with other stimuli including IFNγ+LPS (Fig.4C) and LPS+CD40L (not shown). Supporting the proliferation results we assessed the levels of IFNγ in the

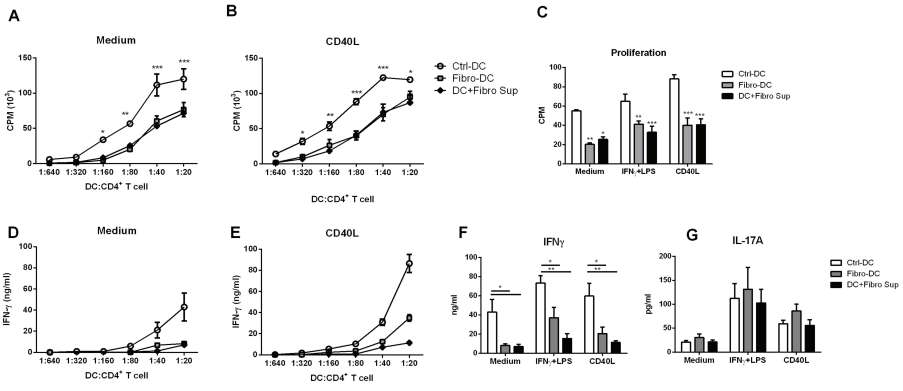

Figure 4: Impaired T cell stimulatory capacity of DCs generated in the presence of renal fibroblasts. Cells were harvested on day 6 and either (A) unstimulated or stimulated with (B) CD40L for 24hrs. The DCs were then harvested and co cultured with allogenic $CD4^+$ T cells at a starting ratio of 1:20. On day 5 the T cell proliferation was determined by ^3H incorporation during the last 12-16hrs of culture. Data shown in (A, B) is representative of 3 independent experiments, with (C) showing the mean ± SD of those 3 experiments. Cytokine production was measured using ELISA. T cell culture supernatants were harvested on day 5 and analysed for IFNγ production induced by (D) unstimulated or (E) CD40L stimulated DCs. Data shown in (D, E) is representative of 3 independent experiments, with (F) showing the mean ± SD of those 3 experiments at the 1:20 ratio. T cell culture supernatants were also assessed for IL-17A production production. Data shown in (G) is the mean ± SD of 3 experiments at 1:20 ratio.

T cell supernatant and found that Fibro-DC and DC+Fibro sup conditions were significantly hampered in their ability to induce IFNγ production (Fig.4D,E,F). Despite the significantly reduced T cell proliferation in Fibro-DC and DC+ Fibro sup conditions (Fig.4C) we found that IL-17A levels remained comparable between all conditions (Fig.4G), suggesting that Fibro-DCs specifically diminish Th1 responses while preserving other T helper responses including Th17.

Fibro-DCs produce significantly less IL-12 compared to Ctrl-DC, yet maintain IL-23.

The IL-12/IL-23 axis is integrally involved in the maintenance of Th1 and Th17 responses respectively. Therefore we investigated the production of IL-12 and IL-23 in both Ctrl-DC and Fibro-DC populations. In all cell types the relatively low mRNA expression of *IL12B* (Fig.5A), *IL12A* (Fig.5B) and *IL23A* (Fig.5C) was strongly increased upon activation with IFNγ+LPS or CD40L. In these activated conditions the mRNA expression between Ctrl-DC, Fibro-DC and DC+Fibro sup was comparable. However, remarkable differences were observed when analysing protein expression. Particularly, activation with IFNγ+LPS induced a strong production of IL-12p40 which was significantly reduced in activated

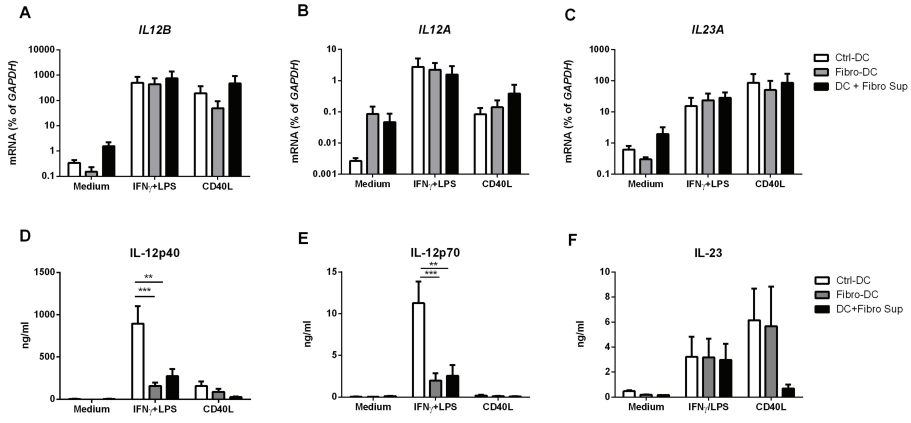

Figure 5: Fibro-DCs have profoundly reduced levels of IL-12p40 and IL-12p70 in cellular supernatants while IL-23 production is maintained. Dendritic cells were harvested after 6 days of culture and stimulated with a combination of IFNγ+LPS or irradiated CD40L cells at a ratio of 1:5 dendritic cells. Cells were stimulated for 6hrs after which mRNA was isolated followed by cDNA synthesis. The transcript levels of (A) *IL12B*, (B) *IL12A* and (C) *IL23A* were determined by RT-PCR. *GAPDH* mRNA expression from the same samples was used as an endogenous reference gene (relative mRNA expression). Data shown is mean ± SD of 3 independent experiments. Cells were stimulated for 24hrs after which the cellular supernatants were collected and assessed for levels of (D) IL-12p40, (E) IL-12p70 and (F) IL-23. Data shown is mean ± SD of 3-8 independent experiments. Untransfected L cells were used as a control for CD40L cells and yielded results in line with medium conditions (data not shown).

Fibro-DCs or DC+Fibro sup (Fig.5D). IL-12p40 can dimerise with either IL-12p35 or IL-23p19 to yield IL-12p70 and IL-23 respectively. We found that IL-12p70 production was profoundly inhibited in Fibro-DC and DC+Fibro sup compared to Ctrl-DC (Fig.5E). However, this stark inhibition was not evident for IL-23 production (Fig.5F). Although IL-23 was diminished in CD40L stimulated DC+Fibro sup conditions, this was not found to be significant.

Fibro-DCs maintain expression of IL-27 and have enhanced levels of IL-10. IL-27 is another member of the IL-12 family and has been reported to possess some anti-inflammatory properties[28-30]. We assessed by RT-PCR, the subunits Ebi3 and IL-27p28, together making IL-27, in each DC type. Both *IL27B* and *IL27A* expression was comparable between Ctrl-DC, Fibro-DCs and DC +Fibro sup conditions (Fig.6A,B). This was also observed in the cellular supernatants where all cell types had comparable levels of IL-27 (Fig.6C).
As an additional cytokine with regulatory properties, we determined the levels of IL-10 in all three cell populations. *IL10* expression was elevated only in Fibro-DC conditions, and not in cells cultured with fibroblast supernatant alone (Fig.6D,E). A similar profile was found for mRNA and protein analysis.

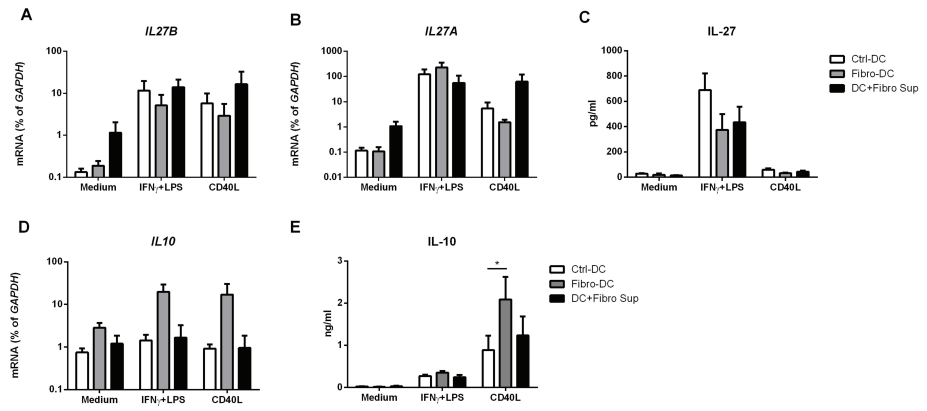

Figure 6: Fibro-DCs maintain expression of IL-27 and have express enhanced levels of IL-10. Dendritic cells were harvested after 6 days of culture and stimulated with a combination of IFNγ+LPS or irradiated CD40L cells. Cells were stimulated for 6hrs after which mRNA was isolated followed by cDNA synthesis. The transcript levels of (A) *IL27B*, (B) *IL27A* and (D) *IL10* were determined by RT-PCR. *GAPDH* mRNA expression from the same samples was used as an endogenous reference gene (relative mRNA expression). Data shown is mean ± SD of 3 independent experiments. Cells were stimulated for 24hrs after which the cellular supernatants were collected and assessed for levels of (C) IL-27 and (E) IL-10. Data shown is mean ± SD of 3-8 independent experiments. Untransfected L cells were used as a control for CD40L cells and yielded results in line with medium conditions (data not shown).

Generation of DCs in the presence of IL-6 can partially mimic the effect of renal fibroblasts.

It became evident to us that fibroblast supernatant alone was sufficient to partially induce the regulatory DC phenotype induced by renal fibroblasts. IL-6 is a pleiotropic cytokine possessing not only inflammatory but also regulatory properties and has been implicated as playing a role in the generation of tolerogenic DCs in vitro[31]. We found that IL-6 was produced under steady state conditions by fibroblasts in culture (not shown), and demonstrated that IL-6 levels were significantly higher in Fibro-DC cultures compared to Ctrl-DC cultures on day 6 (Fig. 7A). Using recombinant huIL-6, we found that DCs generated in the presence of increasing doses of IL-6 were hampered in their ability to produce IL-12, when stimulated with IFNγ+LPS (Fig.7B). This reduced IL-12 was evident when DCs were generated in the presence of just 100pg/ml IL-6 and reached maximum inhibition at 1.0ng/ml (Fig.7B). IL-6 showed a dose dependent increase of IL-10 production, which was minimal at 100pg/ml and continued to increase, with stimulation, until 100ng/ml (Fig.7C). Therefore, it appeared that more IL-6 was required to drive IL-10 production, than was necessary to inhibit IL-12 (Fig.7C). Flow cytometric analysis revealed that CD86 levels were reduced with increasing IL-6 concentrations (Fig.7D). In contrast B7DC and CD1a levels remained

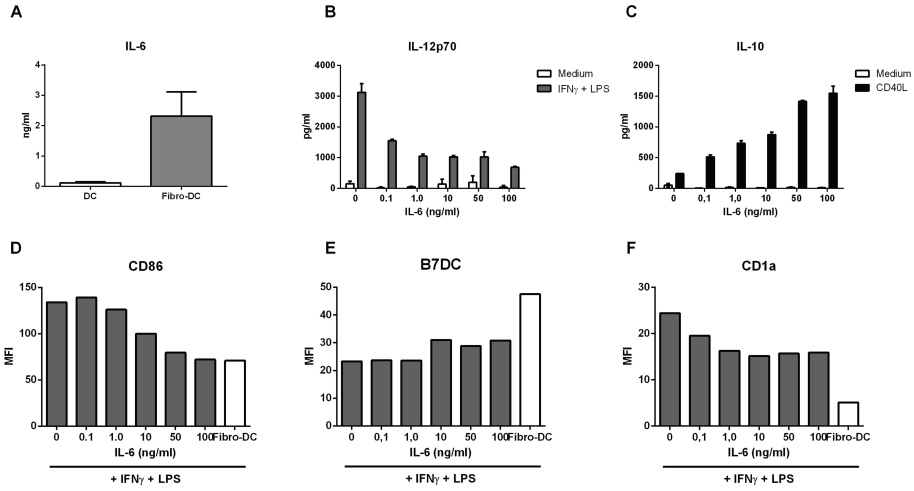

Figure 7: Generation of DCs in the presence of IL-6 can partially mimic the effect of renal fibroblasts. Ctrl-DC and Fibro-DCs were generated as described. Day 6 supernatants were assessed for the presence of (A) IL-6 by ELISA. Monocytes were isolated and cultured with increasing concentrations of rec human IL-6, in addition to GM-CSF and IL-4. Cytokines were refreshed on day 3. On day 6 cells were harvested washed and replated followed by stimulation with IFNγ+LPS or CD40L. After 24hrs the levels of (B) IL-12p70 and (C) IL-10 were assessed. The cells were tested by flow cytometry for the expression of (D) CD86, (E) B7DC and (F) CD1a. Data shown is representative of 2 independent experiments.

unaffected by the increasing doses of IL-6 (Fig.7E,F). These results suggest that like renal fibroblast supernatant, IL-6 is sufficient to reduce the immunogenic phenotype of DCs but is not fully adept at inducing the immunoregulatory properties, including B7DC expression.

Neutralisation of IL-6 in Fibro-DC cultures can partly restore IL-12 production.

The results from the recombinant IL-6 experiments strongly suggested that IL-6 may be a key determinant in how renal fibroblasts induce some of the functional changes we observed in Fibro-DCs (Fig.7). To investigate this, we generated Fibro-DCs in the presence of blocking antibodies against IL-6 or IL-6RA. In line with this we found that IFNγ+LPS stimulated Fibro-DCs demonstrated a strong inhibition of IL-12p40 which was partially reverted when these cells were generated in the presence of either αIL-6 or αIL-6RA (Fig.8A). A similar reversal of inhibition was also observed for the production of IL-12p70 (Fig.8B).

Conversely the increased levels of IL-10 production could be partially reduced when the Fibro-DCs were generated in the presence of either αIL-6 or αIL-6RA (Fig.8C). Flow cytometric analysis showed that reduction of CD86 and CD1a on Fibro-DC was not reversed by αIL-6 or αIL-6RA (Fig.8D,F). However, both αIL-6 and αIL-6RA prevented the increased expression of B7DC on Fibro-DC

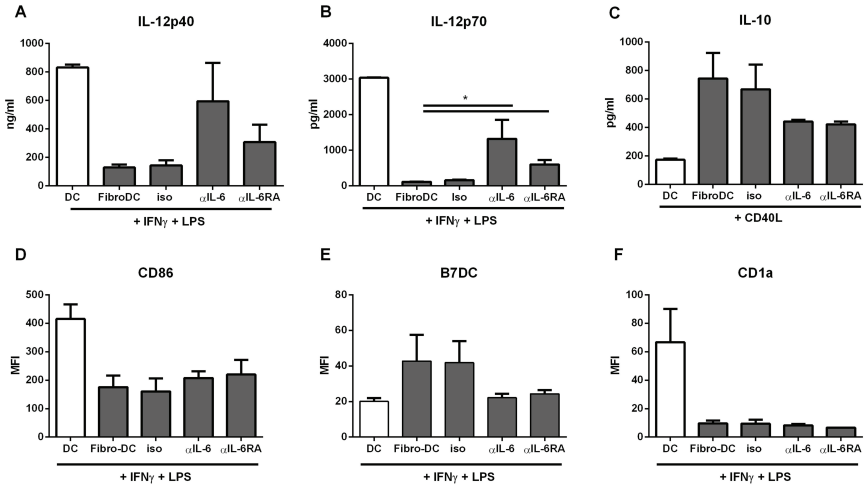

Figure 8: Neutralisation of IL-6 in Fibro-DC cultures can partly restore IL-12 production. Ctrl-DC and Fibro-DCs were generated as described. Antibodies (2.5μg/ml) directed against IL-6, IL-6RA or isotype matched controls, were added to Fibro-DC cultures on day 0 and refreshed daily. Cells were harvested on day 6, washed and replated followed by stimulation with IFNγ+LPS or CD40L. After 24hrs the levels of (A) IL-12p40 and (B) IL-12p70 and (C) IL-10 were assessed by ELISA. Data shown is the mean ± SD of 3 independent experiments. The cells were tested by flow cytometry for the expression of (D) CD86, (E) B7DC and (F) CD1a. Data shown is representative is the mean ± SD of 2 independent experiments.

(Fig.8E).

Discussion

Dendritic cells represent a heterogeneous population of APCs that are found in virtually all tissues of the body, in close contact with the local stromal network [9,32]. In this study we demonstrate that co-culture of DCs with renal fibroblasts, partially through the production of IL-6, leads to the generation of DCs expressing increased immunoregulatory molecules and with diminished allostimulatory capabilities.

In recent years questions have arisen as to whether DCs present in peripheral organs are intrinsically similar because ontogenetically they may be comparable, or are they different because the various local microenvironments dictate the development of tissue specific DCs[32]. Renal DCs (rDCs) form an extensive surveillance network in the human and mouse kidney and are thought to possess immune homeostatic roles involved in inducing and preserving immune tolerance against self and innocuous antigens[33]. Although not fully elucidated, it is believed that local rDCs may exacerbate renal pathology during acute injury, such as ischemia reperfusion injury (IRI)[4].

In the human kidney, fibroblasts and the mononuclear phagocytic system represent a significant proportion of the interstitium[2,17,34]. DCs belong to the latter and we demonstrated that both cells are in close contact in human kidney.

The exact precursor for tissue resident DCs is not known, however we investigated how renal fibroblasts influence the generation of what is typically considered to be the DC precursor during times of stress and inflammation. We found that DCs generated in the presence of renal fibroblasts displayed typical markers including DC-SIGN and MR. Others have investigated the phenotypical changes in DCs cultured with MSCs and noted significantly elevated CD14 expression[35,36]. In some studies it has also been shown that MSCs led to the generation of type 2 anti-inflammatory macrophages[12,37,38]. We assessed the expression of CD163, a typical M2 marker[39,40], and found low expression on both Ctrl-DC and Fibro-DCs (data not shown), indicating that Fibro-DCs are a distinct population compared to that induced by MSCs. Data regarding kidney derived MSCs is more finite, but murine kidney MSCs have been shown to generate a regulatory DC population with decreased MHC II and IL-12 production[41]. Neutralising experiments with IL-6 showed a modest recovery of MHC II while IL-12 restoration was not investigated[41]. In addition, others have investigated the modulation of DCs by human tubular epithelial cells (TEC)[42]. The authors found elevated CD14 and B7H1 with decreased HLA-DR and CD86. Although there appears to be some degree of redundancy in how renal cells influence DCs, TECs are typically not in direct cell contact with renal DCs in vivo, whereas fibroblasts are, suggesting that

these cells may be more directly involved in modulating DC responses.
Comparing immature Ctrl-DCs and Fibro-DCs, we observed that both CD80 and CD86 had a tendency to be higher on Fibro-DCs. This phenomenon, though paradoxical, has also been observed on human tolerogenic DCs[24] and murine BMDCs generated in the presence of kidney MSCs[43]. Upon maturation Ctrl-DCs offered characteristic upregulation of CD80 and CD86 but this increase was significantly reduced on Fibro-DC. In addition, we show for the first time an elevation in B7H1 and B7DC on DCs generated in the presence of renal fibroblasts. Both molecules are accepted markers of regulatory DCs and play an important role in controlling immune responses[44,45].
In line with the stark phenotypical changes, Fibro-DCs were strongly hampered in their ability to induce allogenic T cell proliferation. Notably, although IFNγ levels were reduced in T cells allostimulated with Fibro-DC, IL-17A levels were maintained. It has been previously suggested that the default helper T cell skewing by lung and mucosal tissues is Th17[46,47]. This might explain the observed preservation of IL-17A but not IFNγ, by T cells co-cultured with Fibro-DC. We found that Fibro-DCs profoundly inhibited the production of both IL-12p40 and IL-12p70 when stimulated with IFNγ+LPS. Notably, in line with the IL-17A data, IL-23 production was maintained, despite a reduction in IL-12p40 production. This raises important questions on the regulation of expression, translation and assembly of the IL-12 family of cytokines. It has been suggested that IL-12p35 is more often the rate limiting factor for IL-12p70 production[48,49]. We assessed the transcriptional expression of the entire IL-12 family. Interestingly, although we observed significant inhibition of IL-12p40 at the protein level this was not reflected at the mRNA level, thereby implying there may be some post transcriptional regulation in Fibro-DCs to limit IL-12p40 translation. This has not been seen in other studies as transcriptional regulation has been largely unstudied. Further experiments with fibroblast supernatant and recombinant IL-6 suggested that renal fibroblasts limited the immunogenic properties of DCs via IL-6 secretion. IL-6 has been investigated in studies showing DC modulation by MSCs and stromal cells[11,36,50] and has in recent years been explored for the generation of tolerogenic DCs in vitro[31,51]. However some regulatory phenotypical changes, including B7DC and IL-10 upregulation required cell contact with renal fibroblasts. Neutralisation of IL-6 in Fibro-DC cultures could partly restore IL-12 production, but not CD86 expression, indicating that there may be redundancy and several other mechanisms could potentially be involved in the regulation of DC function by renal fibroblasts. Hepatic stromal cells have been demonstrated to induce regulatory DCs through the action of C3[22]. Additionally, PGE2 has been implicated in MSC mediated DC modulation[52]. We did not investigate PGE2 but did assess the levels of C3 in Ctrl-DC and Fibro-DC cultures, but

did not observe significant differences (data not shown). Nevertheless, local C3 production has been shown in human[53] and mouse[54] kidney, so it may be that even within the organ itself, there are competing or complementary mechanisms of immune homeostasis.

Considering the importance of DCs in immune regulation, surprisingly little is known about the function and regulation of human rDCs, mainly stemming from the caveat that the ontogeny of rDCs is not fully understood. However it is widely thought that monocytes do represent the local DC population during times of tissue stress or inflammation. The majority of the literature on DCs focuses on the contribution of the infiltrating DCs to inflammation in murine pathological disease models. The influence of the local renal stromal cell network within an organ, and its influence on the inflammatory response has been largely unexplored. DCs possess an impressive repertoire of innate PRRs, which is key in helping them decipher the nature of a given insult. Little is known about the expression of such receptors on renal stromal cells, and the function this may play in granting renal fibroblasts a more decisive role in regulating local immune responses.

Summarising, we have shown that DCs cultured in the presence of renal fibroblasts, have a regulatory phenotype, with lower expression of co-stimulatory molecules, and impaired allostimulatory capacity. Production of IL-12 was decreased while IL-23 and IL-10 was maintained or even enhanced in Fibro-DC cultures. These changes are in part mediated through IL-6 secretion from the renal fibroblasts. This study indicates the importance of understanding that the kidney is not only a passive host to DCs and that the local micro-environment plays a major role in maintaining tissue immune homeostasis in health and disease.

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

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