Chapter 5

Non-haematopoietic derived Epstein Barr Virus induced gene 3 expression in normal human kidney.

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Abstract
Epstein-Barr virus-induced gene 3 (Ebi3) is a member of the interleukin (IL)-12 heterodimeric cytokine family, which has important immune-regulatory functions. To date, little is known about the role of Ebi3 in human kidney and in particular its expression in renal DC populations. Considering the abundant expression of Ebi3 in tolDC populations, and the potent regulatory properties of IL-35 we investigated the expression of IL-12 family members within normal human kidney. We examined the expression of Ebi3 in renal tissue by RT-PCR and immune-histochemistry, and found abundant staining for Ebi3 in the glomerulus of normal human kidney. In addition we found markedly high levels of *IL27B* in isolated glomeruli compared to whole kidney. Furthermore, we observed expression of potential chain sharing partners for Ebi3, namely IL-27p28 and IL-12p35 in the glomerular region of normal human kidney. Double staining suggested that podocytes may be the source of Ebi3 in the glomeruli, and further analysis with cultured renal cells demonstrated that podocytes expressed high levels of Ebi3 compared to glomerular endothelial cells and mesangial cells. Stimulation with inflammatory cytokines IFNγ and TNFα, led to a reduction in Ebi3 protein levels in cultured podocytes. We investigated the expression of Ebi3 in an in-vivo inflammatory setting, in 38 paired pre transplant and rejection biopsies, and found reduced transcriptional expression of Ebi3 in the rejection biopsies compared to the pre-transplant samples. Together this data demonstrates the novel expression of Ebi3 by podocytes and suggests a possible unidentified role for Ebi3 in immune homeostasis of the kidney.

Introduction
Since the identification and experimental proof that Dendritic cells (DCs) form an extensive lattice like network within the renal parenchyma[1, 2] the kidney has come to light as expressing surprisingly diverse immune mediators[3]. Tissue resident DCs are thought to be immature sentinels, preserving the immune homeostasis of their environment. During periods of inflammation or stress they protect the host tissue by shaping a local immune response to avoid destructive excessive inflammatory responses and to promote peripheral tolerance[4, 5]. Aside from DCs, resident cells in the kidney, including tubular epithelial cells, have been shown to express several immune molecules including CD40[6], B7H1, ICOS-L[7] and complement factor C3[8]. Additionally renal endothelial cells have been shown to express high levels of MHC class II suggesting that they have the ability to serve as local antigen presenting cells during times of inflammation. Together these findings warrant further investigation into the full immunological contribution of renal cells.
Many cytokines produced locally have been implicated in renal pathologies,

however little is known about local anti-inflammatory mediators that may regulate immune responses. The IL-12 family is composed of 4 cytokines, each related, but possessing distinct functions [9]. Ebi3 can pair with IL-27p28 or IL-12p35 to yield IL-27 or IL-35 respectively. These cytokines represent the regulatory arm of the IL-12 family and both have been shown to exert potent immune-regulatory functions[10, 11]. Expression of the individual chains of the IL-12 family is largely restricted to professional immune cells, particularly DCs and macrophages. Ebi3 however appears to be more broadly expressed in various tissues with its presence demonstrated in placental tissue[12], colonic epithelial cells[13], smooth muscle cells[14] and endothelial cells. Expression of some members of the IL-12 family and their receptors on inflammatory infiltrate have been implicated in the pathogenesis of renal diseases, but to date nothing is known about the expression of the IL-12 family members by the resident cells of the kidney. Considering the abundant expression of Ebi3 in tolDC populations, and the potent regulatory properties of IL-35 we investigated their expression within normal human kidney.

In this study we initially analysed the expression of Ebi3 and related partners, IL-12p35 and IL-27p28, within human kidney with a particular focus on the interstitial areas where renal DCs reside. Instead we found abundant expression of Ebi3 and IL-12p35 within the glomerular region. In-vitro experiments strongly suggested that podocytes were the source of this abundant Ebi3 expression, and that stimulation with inflammatory stimuli can down-regulate Ebi3 expression within this cell population. Analysis of an acute rejection patient cohort demonstrated that compared to the pre-transplant biopsies, Ebi3 transcripts were significantly down-regulated during acute rejection. Together this study demonstrates for the first time, the expression of Ebi3 within normal human kidney and the observation that Ebi3 expression is reduced during renal allograft rejection. These data suggest a possible role for Ebi3 and Ebi3-related cytokines in allograft rejection that requires further investigation.

Materials and Methods

Cell Culture

Conditionally immortalised podocytes (a kind gift of prof P Mathieson)[16] were grown in DMEM medium supplemented 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen, Breda, The Netherlands), insulin (5 µg/ml), transferrin (5 µg/ml), selenium (5 ng/ml) (all Sigma, Zwijndrecht, The Netherlands) and 10% foetal calf serum (FCS). Conditionally immortalised glomerular endothelial cells (GEnC) (a kind gift of prof P Mathieson)[17] were cultured in endothelial growth medium 2 (EGM2 Bulletkit, Lonza) containing 10% FCS and growth factors as supplied. For podocytes and GEnCs, cells were grown at the permissive temperature of 33°C until confluent at which point they were trypsinised and reseeded in fresh flasks at a dilution of between 1:3 and 1:5. The cells were grown to 70 to 80% confluence before thermo switching to 37°C to inactivate the SV40 T antigen and facilitate differentiation. At both temperatures, cells were refreshed with medium 3-4 times per week. Adult mesangial cells (AMC)[18] were cultured in DMEM supplemented with 5% FCS at 37°C. Immortalised human

renal PTEC (HK-2)[19] were grown in serum-free DMEM-F12 (Bio-Whittaker, Walkersville, MD) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, insulin (5 µg/ml), transferrin (5 µg/ml), selenium (5 ng/ml), triiodothyronine (40 ng/ml), epidermal growth factor (10 ng/ml), and hydrocortisone (36 ng/ml, all purchased from Sigma, Zwijndrecht, The Netherlands). Immortalised human renal fibroblasts (TK173)[20] , characterised by the expression of CD73 and PDGRFβ, were cultured in RPMI 1640 with 10% FCS. Human monocytes were isolated from buffy coats obtained from healthy donors using Ficoll density gradient centrifugation followed by positive selection using anti-CD14 MACS microbeads (Miltenyi Biotech GmBH, Bergisch Gladbach, Germany). DCs were generated and cultured in RPMI supplemented with 10% heat inactivated FCS, 90 U/ml penicillin and 90 U/ml streptomycin (Gibco/ Life Technologies, Bleiswijk, The Netherlands), supplemented with 5 ng/ml GM-CSF and 10 ng/ml IL-4 (Invitrogen/ Life Technologies, Bleiswijk, The Netherlands) as described before [21].

mRNA isolation, cDNA synthesis and RT-PCR

Cells were plated at a density of $0.5x10^6$/ml in 6 well plates and stimulated with IFNγ(100ng/ml) (Peprotech, Germany) or TNF-a (20ng/ml) (R&D systems) for indicated time points. Cells were washed after indicated time points and mRNA was isolated using an RNeasy kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Digestion of genomic DNA was performed by using the on-column RNA-free DNase set. Reverse transcriptase system kit (Promega, Leiden, The Netherlands) was used to synthesise cDNA according to the manufacture's instruction and was stored at -20ºC for further analysis. cDNA was amplified by RT-PCR using primers for *IL12A, IL12B, IL23A, IL27A* and *IL27B* (Table I). SYBR Green qPCR master mix (Bio-Rad, Veenendaal, The Netherlands). *GAPDH* was used as the endogenous reference gene. Data analysis was performed using Bio-Rad CFX Manager Software (Bio-Rad). For each sample, the relative abundance of target mRNA was calculated from the obtained Ct values for the target gene and expressed relative to the endogenous reference gene GAPDH.

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
GAPDH	2597	GAPDH	TTCCAGGAGCGAGATCCCT	CACCCATGACGAACATGGG

Western Blotting

Cells were plated at 0.5×10^6 per well in a 6 well plate and stimulated as indicated for 16 hours. Cells were harvested, washed in ice-cold phosphate-buffered saline and lysed in medium stringency lysis buffer for 30 minutes. The lysates were centrifuged at 13,000 rpm, the supernatants harvested, and protein concentration was determined by a Pierce assay. The samples were diluted in 1x SDS loading buffer, boiled for 5 minutes in reducing conditions, followed by separation on 10% SDS-PAGE gel. Gels were transferred to nitrocellulose membranes, blocked with TBS plus 0.1% Tween-20 (TBS-T) and 3% milk and probed with purified anti-Ebi3 (Abnova, Huissen the Netherlands). After incubation with HRP-conjugated secondary antibody, proteins were detected with super signal ECL system. Blots were stripped and β-actin was used as a loading control using HRP

conjugated anti-β actin (Abcam, Cambridge, UK).

Microscopy

For Light-microscopy paraffin-embedded tissue sections were deparaffinised and rehydrated followed by heat antigen retrieval using proteinase K. Slides were then washed in PBS and endogenous peroxidases were directly blocked with 0,4% H_2O_2 followed by 1% BSA/ 1% NHS in PBS for 30 minutes at RT. Slides were incubated with primary antibodies in 1 % BSA/ 1% NHS in PBS for 1 hour, using mouse anti-human Ebi3 (Abnova) or mouse anti-human Ebi3 (DV.25). Both antibodies were detected using the mouse EnVision+ System-HRP (DAKO, Glostrup, Denmark) for 30 minutes. Slides were counter stained using Mayer's hemalum solution. Cover slips were mounted with Entellan (Merck, Darmstadt, Germany).

For Immunofluorescence staining frozen 4 µm Tissue sections were fixed in cold acetone and endogenous peroxidases were blocked with 0,4% H_2O_2 for 30 minutes at room temperature (RT). Subsequently slides were washed and blocked with non-specific serum (1% heat-inactivated normal human serum (NHS)/ 1% bovine serum albumin (BSA) in PBS) for 30 minutes at RT. Slides were then incubated with indicated primary antibodies in 1% NHS/ 1% BSA in PBS overnight at RT, washed and incubated on the next day with secondary Alexa^{568} for 1 hour at RT. Nuclei were counterstained with Hoechst. Non-conjugated mouse isotype antibodies were used to determine the level of non-specific background staining. Sections were mounted with DABCO glycerol (Sigma, Uithoorn, The Netherlands).

Statistical analysis

Statistical analysis was performed with GraphPad Prism (GraphPad Software, San Diego, CA) using a one-tailed or two-tailed t-test as described. P-values ≤ 0.05 were considered statistically significant.

Results

Ebi3 is highly expressed in the glomeruli and distal tubules of normal human kidney.

Considering the potent immuno-modulatory functions of Ebi3 related cytokines we assessed the expression of the Ebi3 in normal human kidney. We performed immunohistochemical staining in both frozen and paraffin sections of the cortex of normal human kidney and observed an abundant expression of Ebi3 staining in the glomeruli and Bowman's capsule. We found little expression of Ebi3 present within the interstitial spaces, however we did observe a strong Ebi3 staining within a proportion of tubuli (Fig.1A). We confirmed this staining using two different antibodies, and found a comparable pattern of expression between both antibodies (Fig.1A). Staining for Ebi3 on frozen tissue demonstrated similar staining to that observed in paraffin tissue, with a strong Ebi3 signature observed in the glomerular region (Fig.1B). Incubation of sections with isotype-matched control antibodies showed no reactivity, thus confirming the specificity of the detected signals. mRNA was isolated from the glomeruli of normal kidney or whole kidney (paired samples) and analysed for the expression of *IL27B*. Although *IL27B* was expressed in whole kidney, in line with the immunohistochemistry findings, the glomerular mRNA fraction showed significantly higher levels of

IL27B expression (Fig.1B). These data suggest that Ebi3 is produced in normal human kidney and may exist as a homodimer, or as a heterodimer to form IL-27 or IL-35.

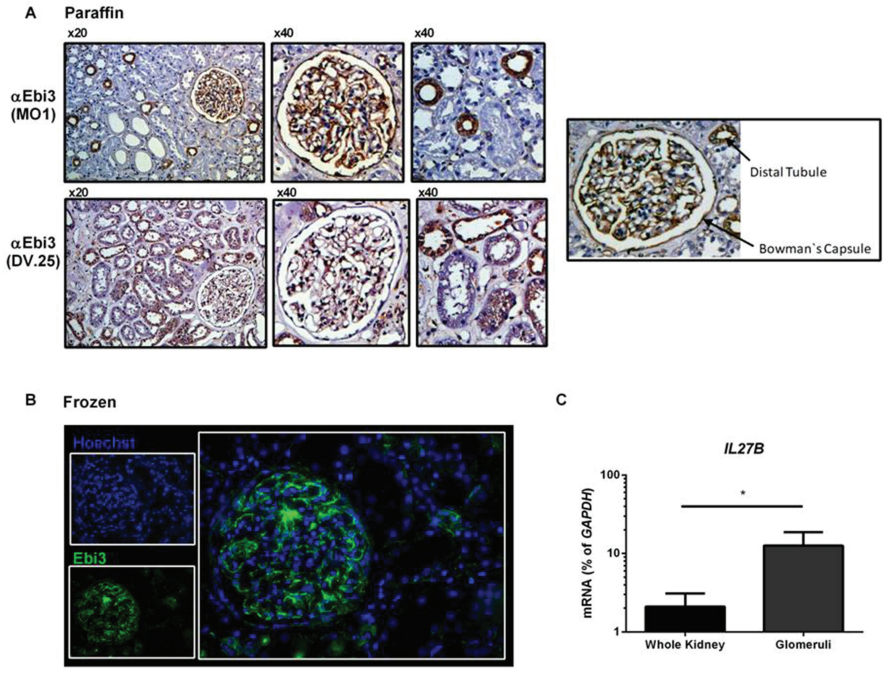

Figure 1: Ebi3 is highly expressed in the glomeruli and distal tubules of normal human kidney. Sections of normal human kidney were stained for Ebi3 in (A) Paraffin embedded tissue. (B) Immunofluorescent staining was performed on cryosections (4μm) of normal human kidneys for Ebi3 (green). Nuclei were 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. (C) mRNA was isolated from glomeruli or whole kidney from different donors followed by cDNA synthesis. The transcript levels of *IL27B* 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 (* $p \leq 0.05$).

IL-12p35 and IL-27p28, potential pairing partners for Ebi3, are both expressed in normal human kidney.

Considering the abundant expression of Ebi3, particularly within the glomerular regions of NHK (Fig.1), we sought to investigate if the potential pairing partners for Ebi3 were also present. We observed by IHC that in addition to Ebi3, normal human kidney expressed high levels of IL-12p35 and to a lesser degree, IL-27p28 (Fig.2A). Notably IL-12p35 expression demonstrated a comparable staining to Ebi3 within the glomerular region, but expression in tubuli appeared to be in a distinct population than that of the Ebi3 staining in distal tubuli (Fig.2A).

We performed double staining for Ebi3 (red) and IL-27p28 (green) on frozen tissue and found little or no overlap between Ebi3 staining and IL-27p28 within glomeruli (Fig.2B). In contrast when we performed the same staining for Ebi3 (red) and IL-12p35 (green) we observed large areas of overlapping staining within the glomeruli (Fig.2B). Notably we did not see any double staining in the tubular interstitial areas (not shown). To determine the cell type expressing Ebi3 within the glomerular region, we performed double staining for Ebi3 (red) with the C type lectin receptor and podocyte marker, DEC-205 (green), or Ebi3 (red) with a marker for endothelial cells (CD31). We found that staining for both Ebi3 and DEC-205 were often found in close contact within glomeruli suggesting that podocytes are most likely the source of Ebi3 within glomeruli (Fig.2C). In contrast it was evident that Ebi3 and CD31 staining had no overlapping regions within the glomeruli or interstitial areas. The only evidence of Ebi3 and CD31 co-staining was within small capillaries or the inner layer of large vessels, which is in line with previous studies demonstrating Ebi3 expression in vascular endothelial cells (Fig.2C).

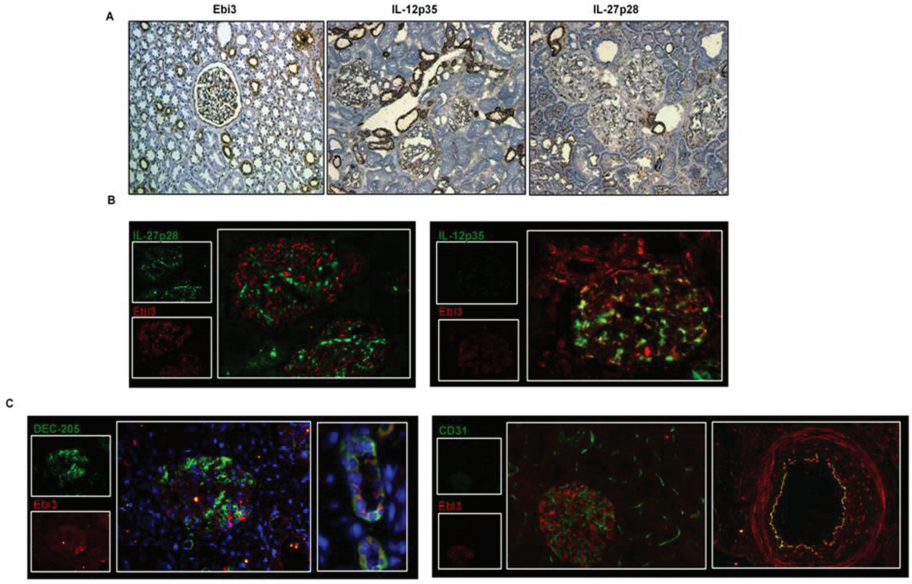

Figure 2: IL-12p35 and IL-27p28, potential pairing partners for Ebi3, are both expressed in normal human kidney. Paraffin and frozen sections of normal human kidney were stained for (A) Ebi3, IL-12p35 or IL-27p28. (B) Immunofluorescent staining was performed on cryosections (4µm) of normal human kidney for Ebi3 (red) and IL-27p28 (green) or Ebi3 (red) and IL-12p35 (green). (C) Immunofluorescent staining was performed on cryosections (4µm) of normal human kidney for Ebi3 (red) and DEC-205 (green) or Ebi3 (red) and CD31 (green). 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.

Analysis of IL-12 family expression in cultured glomerular and interstitial cells.

Given the abundant expression of Ebi3 and IL-12p35 in normal kidney, and the co-staining of Ebi3 with the podocytic marker DEC-205, we sought to further confirm the source of Ebi3 in the kidney by assessing the mRNA transcripts of IL-12 family members in cultured renal cell lines. We cultured glomerular endothelial cells, podocytes and mesangial cells in addition to fibroblasts and tubular epithelial cells. In addition we assessed transcript levels in immature DCs as a representative of professional APCs. We found that transcripts of *IL12A*, *IL27B* and *IL23A* demonstrated the most abundant levels in renal cell cultures with up to 100 fold higher levels of expression compared to *IL27A* and *IL12B* (Fig 3A). While *IL27B* transcripts were detected in all cell populations we observed between 10 and 100 fold higher expression in tubular epithelial cells and podocytes respectively (Fig.3A), which is in line with our IHC observations in (Fig.2). In contrast, expression of *IL12A* was only modestly elevated in cultured podocytes and did not demonstrate the clear expression profile observed with *IL27B*. Notably, expression of *IL12A, IL23A* and *IL27B* in podocytes was

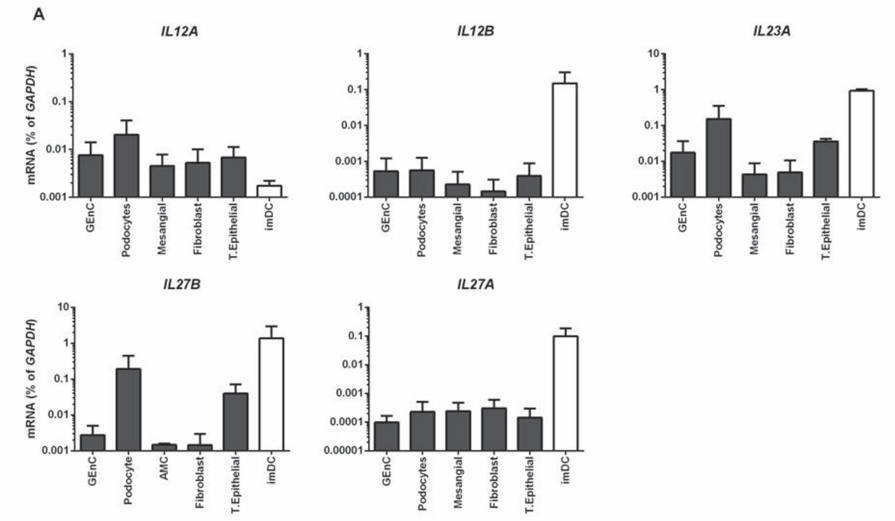

Figure 3: Analysis of IL-12 family member expression in cultured glomerular and interstitial cells. Conditionally immortalised GEnCs, podocytes, mesangial cells, fibroblasts, tubular epithelial cells and immature monocyte dervied DCs were assessed for transcriptional expression of IL27B. GEnCs and podocytes were grown at the permissive temperature of 33°C until confluent at which point they were trypsinised and reseeded in 6 well plates at 0.5×10^6 cells per well before thermoswitching to 37°C for 5 days. All other cell lines were plated in 6 wells plated and together mRNA from the five cell lines and imDCs was isolated followed by cDNA synthesis. The transcript levels of (A) *IL12A, IL12B, IL23A, IL27A* and *IL27B* was 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 2 independent experiments.

relatively comparable with the levels observed in immature DCs, indicating that podocytes may be an important novel source of local IL-12 family members with the kidney.

Podocytes express abundant levels of Ebi3 which is down-regulated upon stimulation with inflammatory cytokines.

It was previously demonstrated that IFNγ + TNFα stimulation led to a synergistic up-regulation of Ebi3 in aortic smooth muscles so we adopted a similar approach by stimulating cells with IFNγ, TNFα or the two combined, for 24 hours (Fig.3A), and found that the stimuli used, exerted differential affects depending on the cell in question. IFNγ alone did not alter the expression of *IL27B* by glomerular endothelial cells whereas TNFα alone or combined with IFNγ led to up-regulation of *IL27B* (Fig.4A). Podocytes demonstrated transcript levels of *IL27B* in line with that found in immature DCs. Podocytes did not demonstrate significant alterations upon stimulation but it did appear that IFNγ down-regulated *IL27B* expression in these cells (Fig.4A). This is contrary to *IL12A* expression whereby

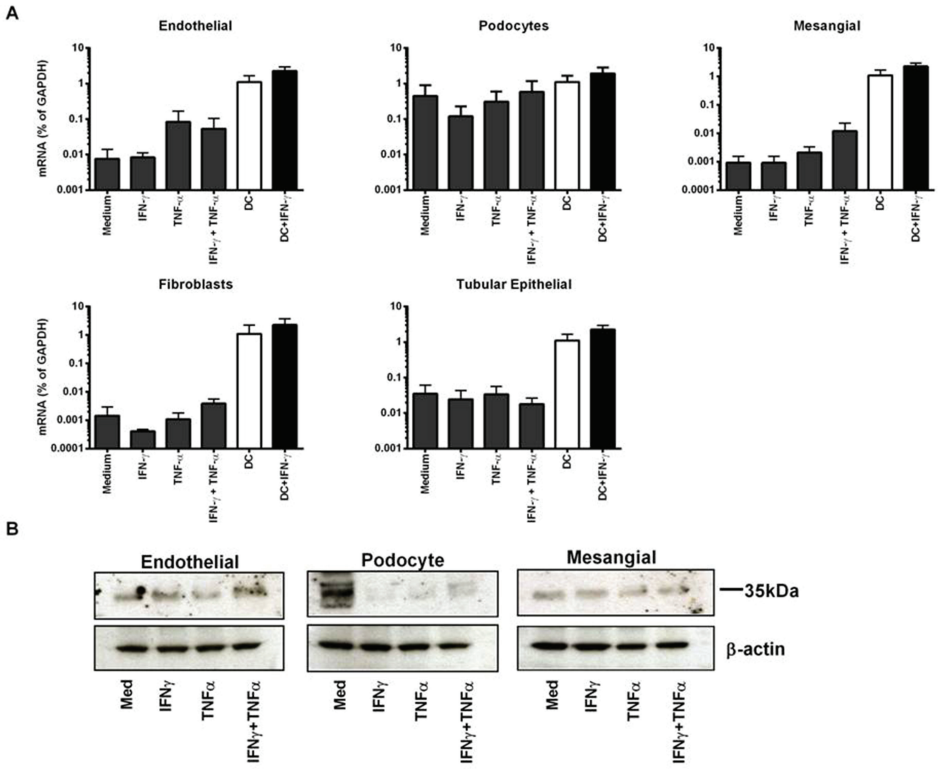

Figure 4: Podocytes express abundant levels of Ebi3, which is down-regulated upon stimulation with inflammatory cytokines. Conditionally immortalised GEnCs, podocytes, mesangial cells, fibroblasts and tubular epithelial cells were assessed for expression of *IL27B* upon stimulation.

GEnCs and podocytes were grown at the permissive temperature of 33°C until confluent at which point they were trypsinised and reseeded in 6 well plates before thermoswitching to 37°C for 5 days. All cells were plated at 0.5×10^6 per well in a 6 well plate and stimulated for 24hrs with IFNγ, TNFα or a combination of both. Additionally imDCs were generated and either unstimulated or stimulated with IFNγ. The transcript levels of (A) *IL27B* was determined in all cell lines and DCs 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 2 independent experiments. (B) Conditionally immortalised GEnCs, podocytes and mesangial cells were assessed for expression of Ebi3 by western blot. Cells were cultured as described for (A). Cells were harvested, washed in ice-cold phosphate-buffered saline and lysed for 30 minutes. The samples were seperated under reducing conditions on 10% SDS-PAGE gel. (B) Gels were transferred to nitrocellulose membranes, blocked with TBS plus 0.1% Tween-20 (TBS-T) and 3% milk and probed with purified anti-Ebi3. Blots were stripped and β-actin was used as a loading control using HRP conjugated anti-β actin. Data shown is representative of 2 independent experiments.

IFNγ stimulation led to upregulation of expression in all cells except fibroblasts (data not shown). Mesangial cells expressed very low transcripts of *IL27B*, and no clear regulation was observed. Similar to podocytes, IFNγ down-regulated *IL27B* expression in fibroblasts, while no clear regulation was observed in tubular epithelial cells (Fig.4A). To determine whether our findings regarding transcriptional expression of *IL27B* corresponded to protein levels, we focussed our studies on the glomerular cells, which demonstrated the most abundant staining in-vivo (Fig.1). We investigated the expression of Ebi3 by western blot upon stimulation with IFNγ, TNFα or a combination of the two (Fig.4B). We found in the unstimulated conditions that, in line with our RT-PCR data, podocytes expressed higher levels of Ebi3 compared to glomerular endothelial cells or mesangial cells. Notably, while both mesangial cells and endothelial cells maintained Ebi3 expression upon stimulation, podocytes demonstrated a reduction. All together these data suggests that podocytes may be the main source of glomerular Ebi3 expression within normal human kidney.

Expression of *IL27B* is significantly down regulated during acute renal allograft rejection.

In view of the abundant expression of Ebi3 expression in normal human kidney and isolated glomeruli (Fig.1), and the modest regulation upon stimulation in immortalised cultured podocytes (Fig.4), we investigated the expression of Ebi3 during an in vivo inflammatory setting namely, renal allograft rejection. From a limited number of available normal human kidney and acute rejection samples we observed that compared to healthy tissue, Ebi3 in the rejection biopsies appeared reduced within the glomerular region (Fig.5A). This was observed across a number of donors. In the normal tissue in (Fig.1) we noted that a proportion of the tubuli also expressed high levels of Ebi3. Additionally Ebi3

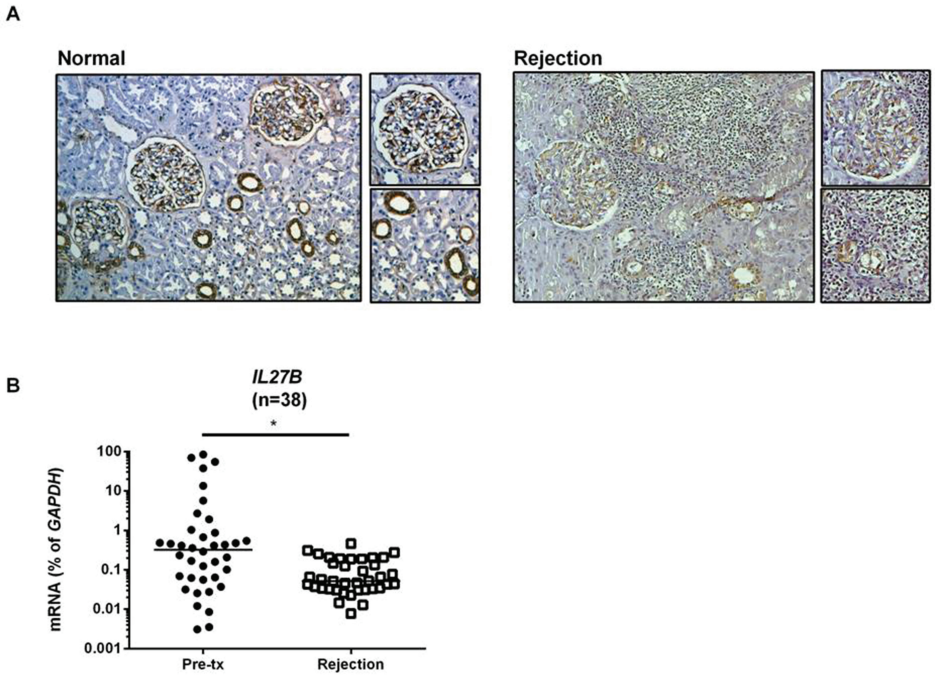

Figure 5: Expression of IL27B is down regulated during acute renal allograft rejection. Sections from normal human kidney and acute rejection kidney were stained for (A) Ebi3 in paraffin embedded tissue. Non-conjugated mouse and rabbit isotype antibodies were used to determine the level of non-specific background staining (not shown). (B) mRNA was isolated from paired pre-transplant and acute rejection tissue from 38 patients followed by cDNA synthesis. The transcript levels of *IL27B* 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 the median level of expression (* $p \leq 0.05$).

has been shown to be expressed by macrophages, DCs and T cells therefore we carefully examined the interstitium and areas of infiltrate for Ebi3 expression. Despite areas of dense infiltrate in rejection biopsies no staining for Ebi3 was evident in these areas (Fig.5A). We further evaluated by RT-PCR the expression of *IL27B* in renal biopsies from 38 patients with a first acute rejection episode. For comparison, the available pre-transplant biopsies (n=38) of these patients were used. Overall, the most striking observation was the wide heterogeneous expression of *IL27B* in pre-transplant biopsies compared to the paired rejection biopsies. In light of the abundant expression of Ebi3 within glomeruli, one should consider that we cannot correct for the number of glomeruli in each sample which may lead to the variability in the pre-tx population. Despite this wide spread in the normal pre-transplant samples, *IL27B* expression was significantly down-regulated in the rejection biopsies (Fig.5B). Out of the 38 patients analysed, 26

demonstrated significant decreases in *IL27B* expression while 12 showed the converse, with increased *IL27B* expression compared to the paired pre-transplant biopsy (Fig.5B).

Discussion

This study describes the first evidence for expression of Ebi3 in human kidney suggesting a role for Ebi3 and related cytokines in the immune-homeostasis of the kidney.

The Epstein-Barr virus-induced gene 3 (Ebi3) is a member of the IL-12 family of cytokines which together are becoming increasingly acknowledged as playing a key decisive role in the maintenance of immunity versus tolerance[21, 22]. The Ebi3 gene was first identified in EBV infected B cells and was found to encode a soluble protein, IL-27β, with structural similarities to IL-12p40[23]. Both IL-12p40 and Ebi3 represent the beta chain subunits of the IL-12 family, and both subunits can pair with two alpha chain subunits within the family. Ebi3 has been shown to form heterodimers with IL-27p28 to yield IL-27, or most recently IL-12p35 to yield IL-35. Both cytokines possess unique immune-modulatory functions, although IL-27 appears to possess dual roles with an ability to also promote Th1 and anti-tumour CTL responses[24-26]. However, a large body of literature has also described the potent regulatory features of IL-27, which amongst others are to promote induction of IL-10 producing Tr1 cells from activated Th1 cells[27, 28], down-regulate Th17 responses[29, 30] and to induce a regulatory phenotype in APCs[31, 32]. IL-35 has been shown to possess profound regulatory abilities and is produced by regulatory T cells in mouse and man and appears to be an important inducible anti-inflammatory cytokine with a significant role in inhibiting Th17 responses[11, 33, 34].

Expression of Ebi3 has been mostly described in haematopoietic cells[23, 35-37] including DCs and we initially sought to investigate the expression of Ebi3 within renal DC populations. However several studies have accumulated data describing expression of this protein in extra-haematopoietic sources including placental synctiotrophoblasts[12], endothelial cells, intestinal mucosa[13, 38] and aortic smooth muscle cells[14]. This expression of Ebi3, often in the absence of its typical pairing subunits has fuelled speculation that Ebi3 may possess biological functions in its own right or may dimerise with as yet unidentified partners.

Despite the extensive DC network that resides within the renal interstitium we did not detect any significant staining that would be indicative of the local DC network. In contrast to TGF-β which has been described as a house keeping anti-inflammatory cytokine[39], IL-35 and IL-27, like IL-10, are most likely not constitutively produced, but rather are inducible upon a particular stimulus. This may be why we did not observe any expression of Ebi3 in the area of resident

DCs. Additionally the cytokine profile of renal DCs is largely unknown, so it is difficult to determine whether these cells would actually be a source of IL-27/IL-35 in vivo. Despite the absence of staining in the interstitial area, we did observe an abundant expression of Ebi3 protein in the glomerulus and Bowman's capsule, and in a proportion of tubuli. We assessed the mRNA isolated from glomeruli from whole kidney of different donors and found a high level of *IL27B* expression within the glomeruli, which was in line with the immunohistochemical staining. Tubular epithelial cells however, are known for their impressive endocytic capacities, so it is difficult to say whether Ebi3 is actively produced by tubular epithelial cells or is endocytosed from the lumen. Further in-situ hybridisation experiments would be necessary to address this specific question. We performed double staining to investigate whether other IL-12 members were also expressed within human kidney and found relatively abundant expression of IL-12p35 and to a lesser degree IL-27p28. In contrast, staining for IL-23p19 was not observed (not shown). Both IL-27p28 and IL-12p35 are known partners for Ebi3 but only IL-12p35 demonstrated an overlapping staining profile with Ebi3 in the glomerular region of human kidney. Investigation into the expression of IL-35 in human kidney has been completely unexplored and further confocal imaging and in-situ hybridisation would be useful in fully confirming the expression of this regulatory cytokine. This would open up further lines of research to understand the role this cytokine may play in renal immune homeostasis and its expression during renal pathology. We also performed double staining of Ebi3 with podocytic and endothelial markers. While we did not observe co-staining with CD31, Ebi3 did appear to stain in regions where podocytes were present. To expand on the findings observed in renal tissue, we cultured cells that are found within the glomerulus and interstitium to investigation the production and regulation of Ebi3 within renal cells. Interestingly podocytes showed the most abundant mRNA and protein expression of Ebi3 indicating that these cells are likely to be the source of Ebi3 staining within the glomeruli of human kidney. Podocytes are highly specialised cells which support the glomerular capillaries and regulate glomerular permeability. Considering podocytes are in close contact with the circulation it is likely that these cells encounter antigen from foreign or noxious sources. More recently studies have shown that podocytes can acquire functions typically attributed to macrophages and Dendritic cells, including expression of CD80 upon LPS stimulation[40], up-regulation and activation of TLR4 leading to the local release of chemokines[41] and even presentation of exogenous antigen in the context of MHC class II[42]. Additionally studies have shown that podocytes are proficient cells at clearing Ig complexes from the glomerular basement membrane[43] and have the ability to ingest and process antigen for presentation[42], together indicating that podocytes are important immunologically active cells

within the kidney. These exciting new revelations warrant further immunological investigation into these novel antigen presenting cells.

Notably the expression of *IL27B* was lower in cultured podocytes compared to isolated glomeruli. Despite the presence of "contaminating" endothelial and mesangial cells in the glomerular isolate, it strongly suggests that primary podocytes may express even higher levels of *IL27B* than we observed with immortalised cells. We had also observed staining for Ebi3 in the bowman's capsule of the glomeruli, where parietal epithelial cells (PECs) reside. It is thought that considering podocytes do not have the ability to self renew, that PECs may migrate and differentiate into podocytes in vivo[44], so it is intriguing to observe the expression of Ebi3 within both cell populations. Upon stimulation with inflammatory cytokines, we found that the podocytes in culture demonstrated a reduction in Ebi3 expression. This in contrast to findings using epithelial and endothelial cell lines from other sources, where it was found that IFNγ, IL-1 and TNFα could significantly enhance Ebi3 expression[13, 14]. It would be informative to investigate the regulation of Ebi3 in primary podocytes to determine if a similar observation occurs. Nevertheless, in light of the abundant expression of Ebi3 in both human kidney and cultured podocytes, we sought to investigate the expression of Ebi3 in an inflammatory renal state. Considering the most important threat to the loss of a fünctioning renal allograft is rejection we analysed the changes in Ebi3 expression in paired pre transplant and rejection biopsies from 38 patients. We first observed by immunohistochemical staining that glomerular Ebi3 expression appeared to be decreased during rejection. Interestingly, staining for the podocytes marker DEC-205 was also reduced in the glomeruar region of rejection biopsies[45] (data not shown), which further supports the hypothesis that podocytes are the source for Ebi3 within the kidney. This was confirmed by RT-PCR and overall we found that Ebi3 expression was significantly reduced in almost 70% of the patients. In summary this study demonstrates the production of the novel IL-12 family member, Ebi3, in human kidney. We provide evidence that podocytes are the likely source of Ebi3 and that inflammatory mediators reduce expression. In addition we describe a reduction of IL27B expression in renal allograft rejection samples compared to the pre-transplant specimen. Together these findings warrant further investigation to fully understand the role of Ebi3 in the immune homeostasis of the kidney and the functional significance of its reduction during rejection.

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