Interleukin-13 is Expressed in Mouse Kidney Allograft Rejection and Mediates Proliferation of Renal Tubular Epithelium *In Vitro*

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Abstract: Kidney transplantation is the preferred therapy for kidney failure. The leading cause of graft loss is chronic allograft nephropathy (CAN). We hypothesize that Interleukin-13 (IL-13), protective against acute kidney graft rejection, is involved in CAN. In mouse kidney allografts, we observe after 2 weeks signs of interstitial inflammation progressing to vasculitis. By 6 weeks, CAN is manifest. IL-13 is overexpressed in allografts versus isografts (p<0.01) throughout the post-transplant course. Concomitantly, we detect markers of fibrogenesis and epithelial-mesenchymal transition. To explore this phenomenon, kidney proximal tubular epithelial cells were cultured with IL-13. Within 6 hours, we show increased proliferation compared to untreated cells (p<0.01), occurring through activation of IL4Rα/JAK3 and Stat6 and blocked by anti-IL-13 monoclonal antibody. This is the first report of IL-13 inducing a specific biological activity in kidney epithelial cells with a possible role in the damage control machinery, indicating its potential as a biomarker and therapeutic target.

Keywords: Kidney transplant, chronic, injury, epithelial cell, IL-13.

INTRODUCTION

Kidney transplantation is the preferred mode of renal replacement therapy, due to advances in immunosuppressive medications that facilitate excellent short term graft survival with low levels of acute rejection. However, long term graft survival in kidney transplantation has not improved substantially [1]. The leading cause of late graft loss is an entity called chronic allograft nephropathy (CAN), characterized histologically by tubular atrophy (TA) and interstitial fibrosis (IF) [2] and may be accompanied by arteriosclerosis and glomerulosclerosis. CAN is characterized by a progressive decline in kidney graft function months to years after transplant, associated with hypertension and proteinuria. Both antigen dependent and independent factors may lead to its development, and the absence of specific treatments for this disorder leads to an eventual need for retransplantation. Identifying new targets to ameliorate this process is of utmost interest in the transplant community.

IL-13 is a pleoitropic cytokine, first discovered to inhibit inflammatory cytokine production [3]. IL-13 is produced by a wide variety of cells including T cells [3-6], natural Killer T cells [7], mast cells, basophils, and eosinophils [8]. While its ability to activate B lymphocytes and stimulate production of IgE and IgG4 is well described [5, 9], recent studies have linked IL-13 to fibrosis in a signal transducer and activator of transcription (Stat) 6-dependent fashion [10]. In both macrophages [11] and dendritic cells [12], IL-13 induces the 'alternative activation' pathway, which leads to the production of arginase type 1 (Arg-1), an enzyme which catalyzes the conversion of L-arginine into proline, and ultimately collagen. IL-13 also induces the production of transforming growth factor beta (TGFβ) in macrophages, indirectly promoting fibrosis [13]. On the other hand, IL-13 was recently described to have a protecting role against acute rejection, as graft receiving IL-13 gene therapy showed a lower rate of injury [14].

Thus, while IL-13 is linked to fibrosis both directly and indirectly, it may also be protective in injury processes. We hypothesize that IL-13 may play a role in chronic allograft injury of kidney allografts with direct effects on renal tubular epithelium. To explore this possibility, we utilized a well characterized mouse model of kidney transplantation that develops acute rejection followed by severe chronic injury. Following kidney transplantation, rejecting allografts have markedly elevated levels of gene expression for IL-13, compared to non-rejecting, syngeneic control grafts and associated with other markers of fibrogenesis, prior to the development of TA/IF. Further, we show that IL-13 mediates proliferation in kidney tubular epithelial cells *in vitro*. These results suggest that IL-13 may induce cell proliferation in the proximal tubules, hence favoring cell replacement and impeding allograft fibrosis and tubular atrophy in kidney allografts. These studies are the first to link IL-13 with late graft failure and present a potential new target to improve allograft survival.

MATERIALS AND METHODOLOGY

Animals

C57BL/6 and (Balb/c X DBA/2)F₁ mice were acquired from Jackson Laboratory (Bar Harbor, ME) or NCI (Frederick, MD) at 6-8 weeks of age. Kidneys from C57BL/6 (isografts) or MHC incompatible (Balb/c X DBA/2)F₁ (allografts) were transplanted into C57BL/6. Mice were kept in

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the specific pathogen-free facility of the National Institute of Health and received standard laboratory chow and tap water *ad libitum* under approved institutional protocols in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Mouse Kidney Transplantation

Vascularized kidney transplants were performed as previously described [15]. Briefly, mice were anesthetized with isoflurane, and the donor kidney, ureter, and half of the bladder were harvested *en bloc*, including the renal artery with a small aortic cuff and the renal vein with a small caval cuff. These vascular cuffs were anastomosed to the recipient abdominal aorta and vena cava, respectively, below the level of the native renal vessels. Total cold ischemic time averaged 30-35 minutes. Donor and recipient bladders were attached dome to dome. The right native kidney was removed at the time of transplantation and the left native kidney was removed 7 days later. Overall surgical mortality was approximately 15%, and there were no significant differences in perioperative mortality between the experimental groups. Mice were sacrificed at 2, 4, and 6 weeks post transplantation for further studies. Six animals were studied in each group. No immunosuppression was given as the grafts have prolonged survival with about 20% survival of all allografted animals at 6 weeks.

Cell Culture

Mouse proximal tubule epithelial cell line (MCT, a gift of Dr. Eric Neilson*)* and mouse macrophage cell line RAW264.7 (ATCC, Manassas, VA) were cultured in Dulbecco's Modified Eagle Medium with 4 mM glutamine and 4.5 g glucose per liter, pH 7.1 to 7.2, (DMEM, Invitrogen) with or without 10% Fetal bovine serum (FBS, Invitrogen).

Quantitative Real Time PCR Analysis *In Vivo*

A portion of the kidney was snap-frozen in liquid nitrogen and processed for RNA extraction using Trizol (Invitrogen, Carlsbad, CA). *In Vitro*: Cell culture experiments were stopped at 6, 24, or 48 hours. The media was removed and cells lysed using Trizol and processed for RNA extraction. For all samples, genomic DNA was removed using DNA-free kit (Ambion, Austin, TX). Each cDNA template for RT-PCR was prepared by first-strand reverse transcription using random primers (First-strand cDNA synthesis kit; Roche, Indianapolis, IN). TaqMan PCR assays was performed in du/triplicate with 10ng total cDNA templates in 96-well optical plates on an ABI Prism 7700 Sequence Detection System following the manufacturer's recommendations (PE Applied Biosystems, Foster City, CA). Murine TaqMan gene expression assays were purchased from PE Applied Biosystems. For each sample, an amplification plot was generated, displaying an increase in the reporter dye fluorescence (ΔRn) with each PCR cycle. A threshold cycle (C_t) value was calculated, which was the PCR cycle number at which fluorescence reached threshold, based on baseline variability data in the first 15 cycles. Delta-Ct for each gene target was obtained by subtracting the Ct value of 18S rRNA from that of the gene target. Finally, the level of mRNA expression relative to expression in isografts was obtained by the following equation:

Relative expression $= 2^{-(dCt\ of\ target\ in\ treatment\ group-dCt\ of\ target\ in\ normal\ tissue)}$

Polymerase Chain Reaction

PCR was performed using the HotStarTaq kit (Qiagen, Valencia, CA), following the manufacturer's recommendations. Primers for IL-13Ralpha1 were designed from the full cDNA sequence (NM_133990) using Oligoperfect designer (Invitrogen) to obtain a 1105 bp long product. Forward primer**:** T T G G T G C T G C T A C T G T G G A C, reverse primer: C A G G A T C A G G A A T T G G A G G A. PCR products were run on a 1.2% agarose gel and photographed using a Kodak image 1D station (Kodak, New Haven, CT).

Cell Proliferation and Viability

Cells were seeded (50,000 cells/mL) and cultured for 24 hours, then washed with medium without serum and kept 24h in a serum depleted environment. Cell proliferation was measured by following BrdU incorporation using a colorimetric ELISA test (Roche). Cells were cultured with BrdU labeling reagent in the appropriate media, the reaction was stopped at different time and the plates processed following the manufacturer's protocol. Signaling studies used mouse rIL-13 (Biosource. Camarillo, CA) with or without Rat anti mouse IL-13 monoclonal antibody (500ng/mL, Sigma, St Louis, IL), Rabbit polyclonal anti TGFβ antibody (175ng/mL, Cell Signaling Technology, Danvers, MA) as well as the inhibitors A-81-01 (1μM, Sigma), Leflunomide (50μM, Sigma) and LY294002 (50μM, Biosource). Oligonucleotide (ODN) were also used in BrdU assays, for which cells were transfected with 300pmol sense or antisense ODN specific to the Stat6 promoter, previously shown to significantly reduce Stat6 protein expression [16]. Transfection was performed using Lipofectamin 2000 (Invitrogen), following the manufacturer's recommendations Cell viability was measured using the Cell Proliferation Kit II (XTT) (Roche). Briefly, the cultured cells were exposed at different times to a yellow tetrazolium salt XTT solution. The salt molecules are cleaved by mitochondrial dehydrogenases into an orange formazan dye. This dye is quantified by a Victor3 plate reader (PerkinElmen, Boston, MA) at 490nm. This assay also reflects proliferation.

TGFβ1 Measurement

Transforming growth factor concentration was measured using the multispecies TGF-β1 immunoassay ELISA kit (Biosource). Medium from cells cultured with or without IL-13 (0.1ng/mL) was isolated at different time points and processed following the manufacturer's recommendations. The resulting colorimetric reaction was quantified using a Victor3 (PerkinElmer) plate reader at 450nm. 3 experiments were performed in duplicate, and the results are expressed as mean ± SEM.

Western Blot Analysis

Cells were lysed in RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA) per the manufacturer's guidelines. Samples were loaded Laemmli Sample buffer (Bio-Rad, Hercules, CA) on a NuPage 4-12% Bis-Tris gel (Invitrogen). Proteins were transferred onto PVDF membrane (GE Healthcare Bio-Sciences, Uppsala, Sweden). The mem-

brane was incubated with Phospho-Stat6 and Stat6 antibody (Cell Signaling Technology, Danvers, MA) and fitting secondary antibodies. Detection was performed with Supersignal West Femto reagents (Pierce, Rockford, IL) and development with a Kodak image 1D station (Kodak). Quantitation of the protein bands was performed using the Kodak 1D imaging analysis software (Kodak). Normalization to protein loading was performed with G3PDH antibody (Chemicon international, Temecula, CA) on the same blot, after membrane-stripping.

Statistical Analysis

Data are shown as mean ± SEM. Two-sided T-test was used for comparison between two groups, with significance defined as $p < 0.05$.

RESULTS

Il-13 mRNA is Highly Expressed in Rejecting Kidney Allografts

As we have previously described [17], fully-MHC mismatched kidney allografts develop acute rejection at 2 weeks (Fig. **1A**) with marked interstitial inflammation consisting of lymphocytes and macrophages (data not shown). This inflammatory injury persists, and by 6 weeks, is accompanied

Fig. (1). Mouse kidney allografts develop acute rejection and subsequent tubular atrophy and interstitial fibrosis. Periodic Acid Schiff staining of 2 week old allograft (**A**) demonstrating marked inflammatory cell infiltration compared to the normal histology of an isograft (**B**). At 6 weeks, allografts demonstrate ongoing inflammation and tubular atrophy and interstitial fibrosis (**C**), compared to the relatively normal isografts (**D**). Magnification: 200x. Gene expression for IL-13 in allografts at 2 weeks (open bars) and 6 weeks (solid bars) determined by real time PCR. Results are expressed relative to isografted kidney as mean ± SEM (n=4). *p<0.05; ¶ p<0.01; ‡p<0.001 compared to isografted kidneys.

by tubular atrophy and interstitial fibrosis (Fig. **1C**). In contrast, isografts retain relatively normal histology throughout the post transplant period (Fig. **1B** and **1D**). Accompanying these histological changes is a dramatic elevation in intragraft gene expression for IL-13 (Fig. **1E**).

At 2 weeks, expression in allografts is markedly upregulated ($1.8 \times 10^5 \pm 0.6 \times 10^5$ fold) compared to isografts (p<0.01), and remains elevated through 6 weeks ($6.2 \times 10^5 \pm 1.5 \times 10^5$ fold; p<0.01). Moreover, there is associated increased expression in allografts at both 2 and 6 weeks of Phoxp47 (18.4 ± 3.4-fold and 31.2 ± 6.0-fold, respectively; p<0.01), a marker of reactive oxygen species production, PAI-1 (6.6 ± 0.9-fold and 9.2 ± 2.1-fold, respectively, p<0.01), fibronectin (5.3 ± 1.4-fold; p<0.05 at 6 weeks) and collagen Iα1 (4.8 ± 0.6-fold and 14.4 ± 3.1-fold; p<0.01), markers of fibrosis and S100A4 (9.8 ± 2.5-fold and 23.7 ± 5.0-fold; p<0.01), a marker of epithelial-mesenchymal transition, confirming previous findings [17]. Thus, IL-13 gene expression is induced in rejecting kidney allografts concomitantly with markers indicating a pro-fibrotic milieu.
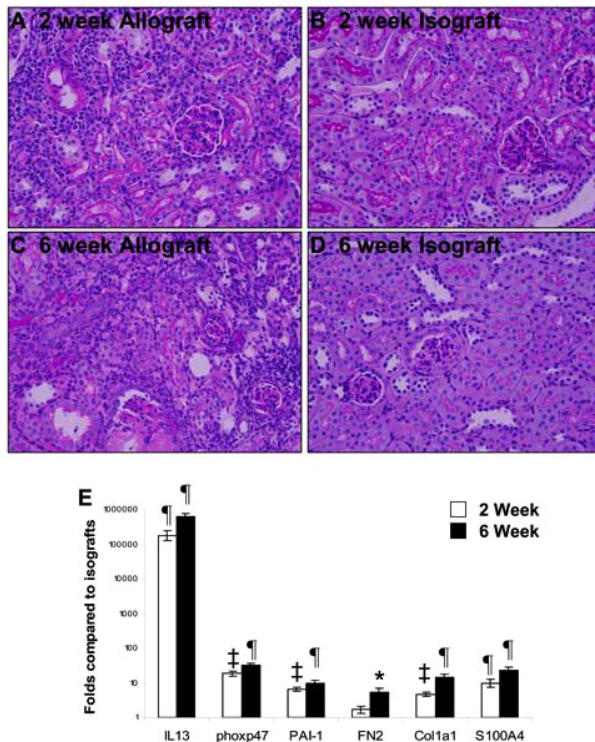
IL-13Rα1 is Highly Expressed in Renal Tubular Cells

To begin to understand the potential role of IL-13 in kidney allografts, we investigated the actions of IL-13 on tubular epithelial cells, one of the primary targets of immune-mediated injury [18]. To determine if tubular epithelial cells had any response to IL-13, we first evaluated for expression of IL-13Rα1, the isoform of the IL-13 receptor that is the known initiator of IL-13 signaling [10], in proximal tubule epithelial cells (PTECs) using the MCT cell line. RAW264.7, a macrophage cell line, was used as a positive control. As shown by agarose gel electrophoresis following PCR, PTECs cells express mRNA for the α1 isoform (Fig. **2A**). To determine the extent of expression, we also performed Taqman RT-PCR, calculating IL13Rα1 expression in RAW cells relative to the expression level in PTECs. As shown in Fig. (**2A**), the level of IL-13Rα1 in PTECs is similar to the levels in RAW cells. Thus, renal tubular epithelial cells express the IL-13Rα1 and have the capacity to respond to IL-13.
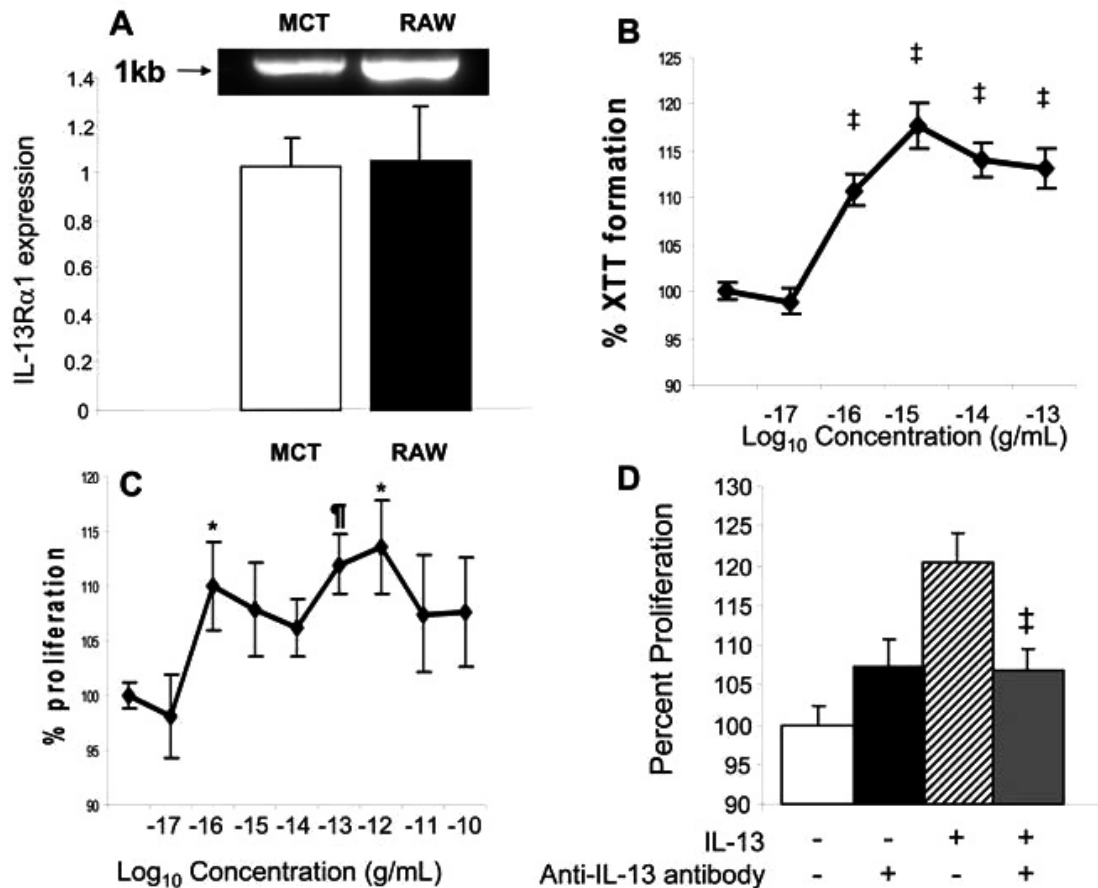
IL-13 Treatment Promotes PTEC Mitochondrial Activity and Proliferation

Following transplantation, epithelial cells are affected not only by ischemic injury but immune mediated injury by lymphocytes. The impact of IL-13 on this process is not known. We thus investigated the effect of IL-13 in cultured PTECs. Small doses of IL-13 (0.01fg/mL to 0.1pg/mL) were added to serum deprived PTEC cells. At the lowest dose of 0.1 fg/mL of IL-13, after only 6h of stimulation, XTT cleavage increased by $110.8 \pm 1.8\%$ of control, (p<0.001; Fig. **2B**), indicating enhanced viability and/or proliferation. To confirm that the XTT cleavage indicates increased cell proliferation, we performed a BrdU incorporation assay over a wide range of dilutions (Fig. **2C**). At 6 hours of IL-13 treatment, PTEC proliferation rate increased by 10% at 0.1fg/mL, peaking at $113.5 \pm 4.37\%$ of control (p<0.05) at 1pg/mL.

To determine the specificity of this response to IL-13, we measured proliferation by IL-13 with and without monoclonal antibody directed against mouse IL-13 (Fig. **2D**). While PTEC basal proliferation was unaffected by IL-13

Fig. (2). Expression of IL-13Rα1 and response of tubular epithelial cells to IL-13 treatment. Gene expression for the receptor is shown by agarose gel electrophoresis (A, top) and by real-time PCR (A, bottom panel) in both resting PTEC proximal tubular epithelial cells and RAW macrophages. Results are expressed as folds over PTEC as mean ± SEM (n=3). B. XTT viability assays on PTEC cells cultured in DMEM + 10% FBS for 6 hours with a gradient of IL-13 (0.01fg/mL to 0.1pg/mL). Assays were conducted as described in material and methods and are results expressed as mean ± SEM for 6 separate experiments. There is an increase in cell viability with IL-13 stimulation, starting at 0.1fg/mL. C. Cell proliferation assay measured by BrdU incorporation on PTEC cells stimulated with increasing concentrations of rIL13, according to methods. Results are expressed as mean ± SEM for 6 separate experiments. IL-13 increases cell proliferation by approximately 10% with exposure to 0.1fg/mL of rIL-13 to a maximum of 13% with 1pg/mL. D. BrdU incorporation assay on PTEC cells comparing resting cells (open bar) with cells stimulated for 6 hours with 0.1pg/mL rIL13 (IL13) with or without 50pg/mL rat anti mouse IL-13 antibody (IL13 mAb). A total of 6 experiments were performed and the results are expressed as the mean ± SEM. Following exposure with rIL-13, there was a 20% increase in proliferation compared to resting cells. This effect was completely abrogated by incubation with anti-IL-13 antibody. *p<0.05; ¶ p<0.01; ‡p<0.001.

specific monoclonal antibody (107.2±3.6% of control), the induction of proliferation with IL-13 (120.5±3.5% of control) was almost completely blocked by co-incubation with anti-IL-13 antibody (106.8±2.8% of control).

IL-13 Induced-Proliferation is Independent of TGFβ Production

As IL-13 is known to induce transforming growth factor beta (TGFβ) production [13], we next measured the production of TGFß in the culture supernatant of PTECs. At 2 hours, 262.8±38.9 ng/mL of active TGFβ was measured in the supernatant. At 48 hours, PTECs continued to produce substantial levels of TGFβ (556.0±15.7ng/mL; p<0.01 compared to 2 hours). Exposure to IL-13 treatment (0.1ng/mL) for 48 hours resulted in 544.0±9.0 ng/mL TGFβ measured,

not different from untreated cells. To further explore the dependence of proliferation on TGFβ expression, cells were cocultured in the presence of A-81-01, a specific inhibitor of ALK5, a component of the TGFβ receptor complex. Based on BrdU incorporation, incubation with A-81-01 did not mitigate IL-13 driven proliferation (125.3±6.1% of control) compared to the level of proliferation mediated by IL-13 alone (123.6±4.7% of control; p=NS). Similarly, neutralization of TGFβ in the media through the use of a polyclonal antibody specific to TGFβ (all isoforms), as described in the methods, had no effect IL-13 mediated proliferation (124.4±5.1% of control) compared to treatment with IL-13 alone (125.3±6.1% of control). Thus, in renal tubular epithelial cells, IL-13 induced proliferation appears independent of TGFß production.

IL-13 Signaling Through its Receptor

The signaling pathway for IL-13 is complex and includes the primary receptor, IL-13Rα1, which once bound induces recruitment of IL-4 receptor alpha 1 (IL4Rα1), forming a high affinity heterodimer called IL-13R [19-21]. Heterodimerization induces the activation of Janus kinases linked to each chains (TYK2, JAK1 and JAK3), activation and translocation of Stat 6 [7, 22]. Additionally, IL4Rα1 on activation may activate the p180 unit of phosphoinositol-3 kinase (PI3-kinase) [23].

To assess whether a unique signaling pathway exists in renal tubular epithelium, we analyzed inhibitors of signaling pathways for known IL-13R signaling pathways. To determine if heterodimer signaling was present, we exposed cells to leflunomide, a known inhibitor of IL4Rα and JAK3 [24]. As shown in Fig. (**3A**), treatment with 50 µM leflunomide reduced IL-13 induced PTEC proliferation by 40±5.5% (p<0.001) compared to IL-13 alone. Blockade of PI3-kinase using 50 µM of the specific inhibitor LY294002 (Fig. **3A**) resulted in a reduction of 20±7.9 % in proliferation (p<0.05) in IL-13 stimulated PTEC cells. Thus, IL-13 induced proliferation is dependent on heterodimer IL-13Rα1/IL-4Rα complex, with activation of P3-kinase.

To test the activation and translocation of Stat-6, phosphorylated Stat-6 was measured by western blot. When exposed to 0.1pg/mL IL-13, Stat-6 phosphorylation increased by 140.7±16.4% of control (p<0.05) at 15 min and by 132.6±8.7% of control at 60 minutes (p<0.01). The functional relevance of this activation was further assessed by an oligonucleotide (ODN) strategy [16]. First, antisense ODN reduced the Stat6 protein levels to 69.9±5.6% of control, p<0.001 of control over three independent experiments (Fig. **3C**). Moreover, while sense ODN did not affect the level of IL-13 stimulated PTEC proliferation (Fig. **3D**), incubation with antisense oligonucleotide inhibited IL-13-induced proliferation by 15.8+/-0.5% (p<0.001). Thus IL-13 induces Stat6 phosphorylation, a protein without which IL-13 induced proliferation is not possible. These results show that IL-13 exercise its proliferative effect on PTECs through its described signaling pathway: IL4Rα/JAK3, PI3-kinase and Stat6.

IL-13 does not Mediate Epithelial-Mesenchymal Transition (EMT) of PTECs

To address whether IL-13 may directly initiate a profibrotic response *via* the PTEC expressed receptor, we evaluated gene expression for a number of molecules associated
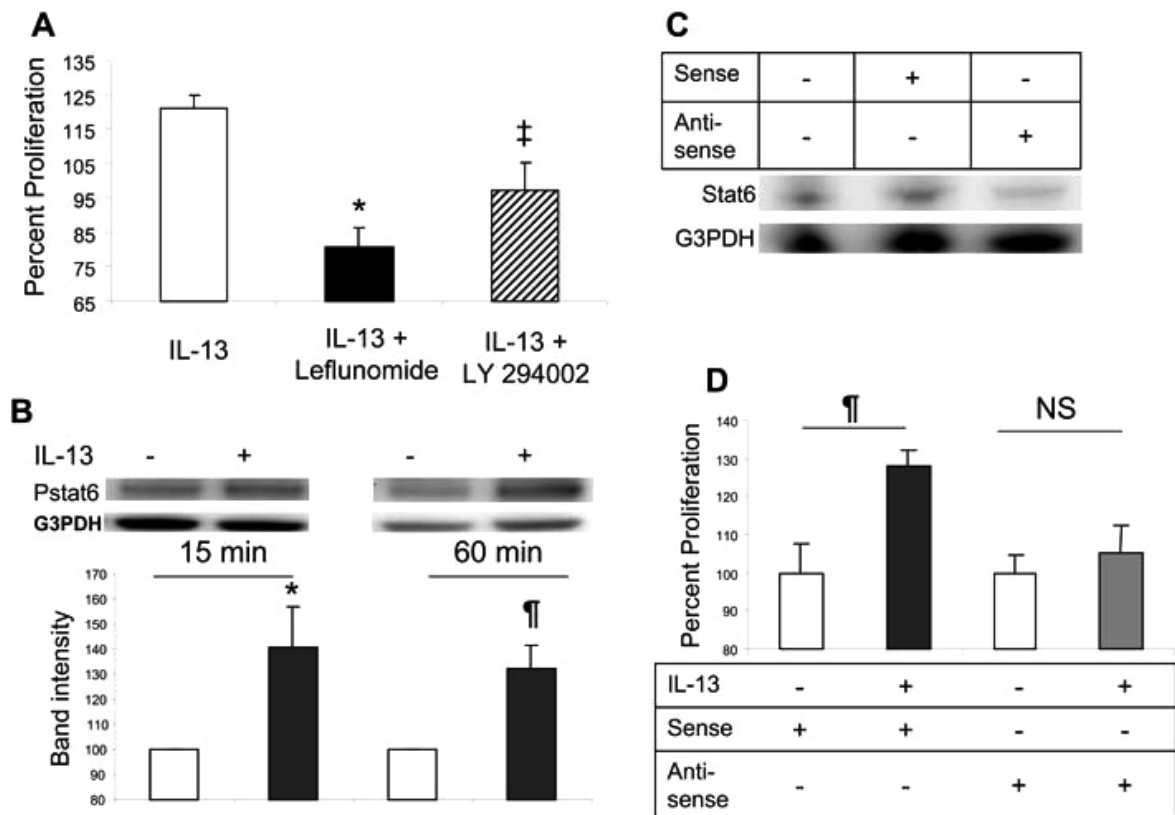
Fig. (3). IL-13 signals through standard IL-13R signaling pathways in renal tubular epithelial cells. In A, cell proliferation measured by BrdU incorporation assay on PTEC cells stimulated for 6 hours with 0.1pg/mL rIL13 (IL13) in the absence or presence of leflunomide or PI3-kinase inhibitor LY294002. Results shown are mean +/- SEM of 12 experiments. There was a marked suppression of IL-13 induced proliferation with both of these agents. B. Representative blot with Phospho-Stat6 (Pst6) staining (B, top lanes) and Glyceraldehyde 3 Phosphate Dehydrogenase (G3PDH) as a control (B, bottom lanes). Signal quantification by Kodak Image 1D station of 3 independent experiments is shown below. IL-13 induces Stat6 phosphorylation 15 and 60 minutes following exposure on PTEC cells. In C, depletion of Stat6 protein level in PTECs by transfection of an antisens nucleotide is demonstrated by western blot. BrdU incorporation of PTEC transfected with a sense ODN or an antisense ODN against the Stat6 promoter, with or without 20ng/mL IL-13 (panel D). Antisense transfection abrogated IL-13-induced proliferation while sense had no effect. *p<0.05; ¶ p<0.01; ‡p<0.001.

Table 1.   Gene Expression Analysis in PTECs Following 20ng/mL IL-13 Exposure

Genes	6 hours culture			24 hours culture			48 hours culture		
	Control	**IL-13**	**P**	**Control**	**IL-13**	**P**	**Control**	**IL-13**	**P**
TGFß	1.2±0.3	1.2±0.6	0.89	1.3±1.1	0.5±0.3	0.10	1.2±0.7	1.2±0.4	0.99
CTGF	1.2±0.4	1.7±0.7	0.42	1.1±0.4	1.5±0.7	0.16	1.1±0.6	1.5±0.3	0.21
S100A4	1.2±0.4	1.0±0.2	0.60	1.0±0.4	2.8±1.5	0.02	1.1±0.4	1.3±0.4	0.41
IGF1	1.1±0.3	1.6±0.3	0.10	n/d	n/d	n/d	n/d	n/d	n/d
αSMA	1.1±0.3	1.8±0.5	0.07	1.0±0.4	0.9±0.8	0.79	1.5±1.3	2.5±3.3	0.58
Vimentin	1.2±0.4	1.3±0.3	0.70	1.1±0.7	5.6±2.9	0.004	1.0±0.2	1.1±0.4	0.69
Fibronectin	1.2±0.4	1.0±0.3	0.62	1.0±0.2	1.1±0.4	0.69	1.0±0.3	0.9±0.2	0.38
E-cadherin	1.1±0.2	2.2±0.9	0.11	1.1±0.5	2.8±1.5	0.02	1.1±0.5	1.3±0.5	0.51
MMP9	1.1±0.2	0.7±0.1	0.05	n/d	n/d	n/d	n/d	n/d	n/d
Bmp7	1.1±0.2	1.5±0.5	0.27	1.3±0.9	1.1±0.8	0.72	1.0±0.2	0.8±0.1	0.11

Results are expressed as fold over expression in untreated control cells (mean±SEM) and a mean of 3 separate experiments. n/d: marker not detected.

with fibrosis and EMT in the kidney. As shown in Table **1**, exposure of PTECs for 6 hours with 20 ng/mL of IL-13 did not induce any significant changes in expression of growth factors CTGF, IGF1 and TGFβ, nor EMT markers S100A4, αSMA, vimentin, E-cadherin, or fibronectin. Negative regulator BMP7 was also unaffected. However, there was a modest but significant decrease in expression in MMP-9 following IL-13 treatment. At 24 hours, expression of EMT markers S100A4 and vimentin were increased significantly, but accompanied also by an upregulation of E-cadherin. However, at 48 hours, these transcriptional differences between PTECs cultured alone or with 20ng/mL IL-13 were not sustained. Thus, IL-13 does not directly mediate a durable profibrotic response in PTECs.

DISCUSSION

The etiology of chronic allograft injury and the development of tubular atrophy and interstitial fibrosis are not clear. At the center of this injury are tubular epithelial cells, which may undergo epithelial-mesenchymal transition (EMT), a critical event in fibrogenesis of kidney grafts [25, 26]. Through this process, tubular epithelial cells may lose their epithelial properties, and adopt a mesenchymal phenotype leading towards cell proliferation, migration out of the tubule, and formation of a pro-fibrotic machinery inside as well as outside of the tubule [26-33]. Real time PCR analysis of the gene expression profile in PTECs stimulated with IL-13 at 24 hours demonstrates upregulation of two markers for EMT but with over-expression of E-cadherin, which is not supportive of EMT. Moreover, these changes are not sustained over time and thus we cannot draw a firm conclusion regarding the direct influence of IL-13 on EMT or fibrosis in these cells. IL-13 impact on proliferation could play a role in the proliferative phase of EMT, therefore accelerating the pace of mesenchymal cell production and potentially aggravating fibrosis. On the other hand, as cell proliferation is integral to recovery of tubular damage, IL-13 may limit the extent of the injury, promoting replacement of dead and apoptotic cells, and thus slowing graft deterioration. The

later is in concordance with recent studies by Sandovici *et al.*[14], which indicate that IL-13 gene therapy of kidney graft could attenuate acute allograft rejection, a phenomenon for which IL-13 driven PTEC proliferation may support. Experiments such as depletion of IL-13 *in vivo* using antibodies [6] or chimeric protein [34] could provide clues towards determining the exact role played by this cytokine. Th2 cytokines have been typically associated with T cell proliferation, stimulation of B cells including immunoglobulin production and the allergic response [35]. While Th2 associated fibrosis has been noted, the mechanism of this response remains unclear and a direct effect of a T cell based cytokine on epithelial cells as we have demonstrated here is uncommon. Recent studies suggest that IL-13 induces fibrosis *via* a TGFβ dependent mechanism in association with TGFβ activation [13]. Our studies did not demonstrate an induction of TGFβ transcription, and this difference may be related to the fact that purified epithelial cells were used whereas prior observations were *in vivo*, with a macrophage-rich milieu. We also tested the effect of IL-4, another Th-2 cytokine which shares a common receptor chain with IL-13, and found a similar potential for inducing proliferation, albeit at a slower pace (24 hours instead of 6 hours, data not shown). Thus Th2 cytokines present within the allograft may not only affect other inflammatory cells within the graft, but may potenciate damage recovery mechanisms by moderating epithelial responses to injury and could thus be contributory to extend the life of the graft. Investigating the influence of the use of immunosuppressive regimes on these processes could permit to better design therapies that are not so detrimental to the long term state of the graft and could potentially harness the power of the immune system towards delaying graft failure.

With the growing interest in non-invasive diagnostic tools such as mRNA analysis of the urine [36] or the blood [37], particularly with high-throughput techniques [38], there is increasing need for new markers of chronic injury. Particularly of interest for diagnostic purposes, these markers need to allow detection of the disease before the process be-

comes irreversible. Herein we demonstrate that IL-13 is detected in the graft several weeks before the development of histological features of the injury, and furthermore we demonstrate *in vitro* the direct influence of this factor on PTECs, increasing their proliferation rate and possibly slowing down the injury process. Consequently, investigating the levels of expression of IL-13 in the blood or urine of patients could provide an early sign of developing chronic injury.

CONCLUSIONS

In kidney transplantation, the leading cause of this graft loss is chronic allograft nephropathy (CAN), histologically characterized by tubular atrophy (TA) and interstitial fibrosis (IF). We demonstrate that in fully-vascularized mouse model of kidney transplantation, allografts show a marked elevation in the gene expression of IL-13 along with markers of chronic injury. Furthermore, we show that *in vitro,* IL-13 induces proximal tubule cell proliferation, suggesting the possible involvement of IL-13 in a mechanisms at the core of damage control during tubular injury. This is the first report of a possible protective biological activity for IL-13 in kidney epithelial cells. Understanding the IL-13 pathway will provide a new diagnostic and therapeutic target for patients with CAN.

CONFLICT OF INTEREST:

The authors have had no involvements that might raise the question of bias in the work reported or in the conclusions, implications, or opinions stated.

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