Published in final edited form as:

J Pediatr Urol. 2020 October ; 16(5): 586–591. doi:10.1016/j.jpuro.2020.05.010.

***Trans* IL-6 signaling does not appear to play a role in renal scarring after urinary tract infection**

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Summary

Introduction: While inflammation is an important innate defense mechanism against infection, it can also lead to local tissue damage. The *trans* signaling pathway of interleukin (IL)-6 is a known mediator of inflammation. We hypothesized that the *trans* IL-6 signaling pathway is associated with the development of post febrile urinary tract infection (UTI) renal scarring.

Objective: To compare soluble regulators of *trans* IL-6 signaling between patients with a history of febrile UTI who do or do not have renal scarring.

Study design: After IRB-approval, we collected urine samples in pediatric patients with a history of febrile (≥ 38°C) UTI (urine culture >50K uropathogen) with documented presence or absence of renal scarring on imaging. Samples were collected at a time when patients were not actively infected. Enzyme-linked immunosorbent assays were performed on samples for markers of *trans* IL-6 signaling: IL-6, soluble (s)IL-6 receptor (R), and soluble (s)gp130, a buffer in *trans* IL-6 signaling. Values were normalized to urine creatinine. Results were analyzed by t-test or Mann-Whitney *U*. Spearman rank correlation was used. A p-value of <0.05 was considered significant.

Results: A total of 50 urines from patients with a history of febrile UTI were collected: 23 with and 27 without scarring. There was no difference between groups regarding age or gender. There was no significant difference in urine IL-6, sIL-6R, or sgp130 between those with and without scarring (Figure). While IL-6 values significantly correlated with sIL-6R and sgp130 in those

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Conflict of Interest Statement: No author has any financial or personal relationships with other people or organizations that could inappropriately influence this work.

Ethical Approval: We had proper IRB approval and consented/assented patients as outlined in this approval.

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without renal scarring, IL-6 did not correlate with sgp130 in those with scarring. Ratios of IL-6 to sgp130 and sIL-6R to sgp130 were not different between groups.

Discussion: The inflammatory response generated in response to infection is believed to be largely responsible for the development of renal scarring after UTI. IL-6 is a cytokine known to be induced during UTI with a pro-inflammatory pathway, known as *trans* signaling. This study investigated for differences in markers of *trans* IL-6 signaling between patients with a history of febrile UTI with and without renal scarring. There was no significant difference between the absolute values or ratio of these markers between groups.

Conclusions: Markers of *trans* IL-6 signaling are not different between individuals with a history of febrile UTI with and without renal scarring in the non-acute setting.

Summary Figure:

Urine sample results of *trans* IL-6 signaling markers in patients with a history of febrile UTI who do not have scarring (NS) compared to those with scarring (S).

Keywords

biomarkers; urinary tract infection; interleukin-6; signal transduction; pyelonephritis

Introduction

Urinary tract infection (UTI) is one of the most common infectious threats to the body, accounting for over 1 million pediatric visits to the emergency department [1, 2]. Our body has developed specific mechanisms to try and address infectious invasion from outside organisms. An important component is the immune system which can help eradicate pathogens. This can come, however, at a cost to the local tissue with resulting local tissue damage, such as in post-pyelonephritic renal scarring. In fact, the development of post-pyelonephritic scarring may depend more on the generated inflammatory response to the bacterial renal infection than be a direct result of the bacteria [3-5].

An important mediator of the inflammatory response is the cytokine interleukin (IL)-6. IL-6 is highly induced during UTI as presumably part of the innate mechanism of response to UTI [6-8]. IL-6 appears to have dual roles in being both protective and deleterious, likely related to at times being anti-inflammatory and others being pro-inflammatory. Explaining this seemingly contradictory role for IL-6 is the fact that IL-6 signals through two different mechanisms: the *cis* and *trans* signaling pathways. As a result, each pathway is associated with a corresponding action: the *cis* or *classic* pathway is regenerative and protective while the *trans* pathway is pro-inflammatory [9, 10]. While there is still uncertainty of what IL-6 induction means for UTI susceptibility, it is particularly unclear what role the various signaling pathways in IL-6 play in the sequela of infection.

This project proposed to evaluate markers of *trans* IL-6 signaling for an association between induction of this signaling pathway with the development of renal scarring after febrile UTI. Our primary outcome was to compare measurable soluble regulators of *trans* IL-6 signaling between patients with a history of febrile UTI who have evidence of renal scarring to those with a history of febrile UTI without scarring.

Materials and Methods

This study was approved by Institutional Review Board (IRB07-00383).

Urine samples from pediatric patients with a history of UTI with and without scarring

Bladder urine samples from consenting/assenting pediatric patients (> 21 years old) being seen in the Pediatric Urology Clinic at our institution for a history of febrile UTI were collected. Febrile UTI was defined as an episode of fever (> 38°C) for which a corresponding urine culture was obtained and grew >50,000 colony forming units of a single uropathogen as per review of the electronic medical record [11]. Time of last UTI and number of prior febrile UTIs were noted as recorded in electronic medical record. Urine samples were collected by free void or catheterization at time of voiding cystourethrogram. Patients were excluded from analysis if they demonstrated signs of active infection on urinalysis (such as positive nitrites or anything more than trace leukocytes) and/or symptomatology at time of urine collection for the purposes of this study.

All imaging obtained as part of the patient's work up for UTI was reviewed including most recent renal ultrasound (RUS) and renal cortical scintigraphy scan using dimercaptosuccinic acid (DMSA), if present. Presence or absence of renal scarring as noted in radiology read was recorded; patients were divided into scarred (S) and non-scarred (NS) patients. If no mention of renal scarring was made by the radiologist read, the patient was included as a NS patient. In order to try and document upper tract involvement during UTI, charts were also reviewed for any imaging at the time of infection that might confirm pyelonephritis.

Sample processing

Urine samples underwent immediate urinalysis by dipstick (Siemens Healthcare Diagnostics, Inc., Tarrytown NY) after collection. They were then placed in a protease inhibitor (Assay Assure, Sierra Molecular, Incline Village NV), cleared by centrifugation,

aliquoted, and stored at -80°C until analysis as previously described [12]. Samples underwent a single freeze/thaw cycle before analysis.

Measurement of trans IL-6 signaling markers

We performed enzyme-linked immunosorbent assays (ELISA) on all urine samples for markers of *trans* IL-6 signaling including IL-6, sIL-6 receptor (sIL-6R), and sgp130 (Duoset ELISA, R&D Systems, Minneapolis MN). IL-6 was measured using undiluted urine, while sIL-6R and sgp130 were both run on urine at 1:15 dilution. ELISAs were performed in duplicate on cell-free supernatants. Results were normalized to urine creatinine (UCr) measured at a 1:10 dilution by colorimetric assay (Oxford Biomedical Research, Rochester Hills MI) and expressed as a ratio to UCr (pg/mg) as previously described [12].

Statistical analysis

D'Agostino-Pearson omnibus normality test determined if samples were parametric. Sample analysis was performed by t-test or Mann-Whitney *U* test depending if they were parametric or nonparametric, respectively. Chi-square test was used to compare categorical variables. Correlation between variables was analyzed by Spearman correlation as comparison groups were nonparametric. A p-value of <0.05 was considered significant. Analysis was performing using GraphPad Prism7.0 (GraphPad Software Inc., La Jolla, CA).

Results

We collected 50 urine samples from children with a history of febrile UTI. Of these, 23 children had evidence of renal scarring on RUS and/or DMSA. The other 27 children did not have evidence of renal scarring on RUS (none of these patients had DMSA imaging). Patient demographics are listed in Table 1. The groups differed by race and number of febrile UTIs. The circumcision status of males was noted. The one male who was uncircumcised in the NS cohort was an infant (0.7 years old) and had documented catheterized urine samples upon work-up for his UTIs. The other circumcised males all were older than 2 years of age and were generally toilet trained. There was a mix of catheterized or free void specimens upon work-up for UTI in those patients.

No patients had DMSAs performed to diagnosis pyelonephritis. Seven patients each in both the NS and S groups had imaging at time of an UTI episode; these consisted of a RUS and/or CT abdomen/pelvis. Three patients in each group had evidence of renal involvement as noted by radiology on their renal ultrasound or CT imaging in the form of renal parenchymal enlargement and patchy echogenicity.

Regarding follow-up imaging, significantly more S children had undergone a DMSA compared to NS children ($p<0.0001$). There was no significant difference between groups regarding those having undergone VCUG and the overall presence of vesicoureteral reflux (VUR) or type of VUR found. Dilating VUR was defined as those with VUR grade 3. The groups did not differ in regard to the grade of VUR included in dilating VUR ($p=0.2036$), with grade 3 VUR being the most common type of VUR in each group (8 patients in NS and 11 patients in S). All S patients but one had matching findings between their DMSA and

RUS. This included even the laterality of the scarring. One patient had a normal RUS both before and after a DMSA showing focal left renal scarring in the lateral midpole.

There was no significant difference in urine IL-6, sIL-6R, or sgp130 between the NS and S groups ($p=0.4859$, $p=0.3855$, and $p=0.2584$, respectively) (Figure 1). In NS patients, sgp130 levels correlated with IL-6 and sIL-6R levels ($p=0.0004$ and $p=0.043$, respectively). In S patients, sgp130 only correlated with sIL-6R ($p<0.0001$), but not with IL-6 (Figure 2). Given these findings, we compared the ratio of IL-6:sgp130 and sIL-6R:sgp130 (Figure 2). These were not significantly different between our two groups ($p=0.4954$ and $p=0.4833$, respectively). In addition, the ratio of IL-6:sIL-6R was not significant ($p=0.5301$).

There was no correlation between IL-6, sIL-6R, and sgp130 levels in children with a history of febrile UTI and gender, method of urine collection, number febrile UTIs, and presence of VUR (all $p>0.06$). There was a correlation between race and IL-6 ($p=0.030$), but not with sIL-6R or sgp130 (both $p>0.8$). There was a correlation with time from last UTI and sgp130 ($p=0.023$), but not with IL-6 or sIL-6R (both $p>0.09$). There was a correlation between age and sIL-6R ($p<0.0001$) and sgp130 ($p=0.043$), but not with IL-6 ($p=0.264$).

Discussion

IL-6 has been shown to have dual effects of inducing and inhibiting inflammation. Likely this balance is important to incite an appropriate immune response to limit infection such as in UTI, as demonstrated by mice that do not express IL-6 having more severe UTI [13], without leading to increasingly severe presentations of UTI [7, 14]. IL-6 signals through two pathways, explaining the diversity in its effects: the *cis* and *trans* pathways. The *cis* or classic pathway is felt to be anti-inflammatory and the *trans* pathway pro-inflammatory. The pathways differ in the location of the IL-6R that binds to circulating IL-6 and the resulting cell susceptibility and downstream signaling cascade. In the *cis* pathway, the IL-6R is bound to the cell membrane in conjunction with another transmembrane protein, gp130, which acts as a signal transducer. IL-6 thus binds to the membrane bound IL-6R and gp130 to initiate biological processes. Membrane bound IL-6R is expressed in only a limited group of cells, including hepatocytes, neutrophils, monocytes, certain leukocytes, and some epithelial cells. However, the IL-6R can be proteolytically cleaved from the membrane to become solubilized (sIL-6R); or sIL-6R can be a result of alternative splicing. In the *trans* pathway, IL-6 binds to sIL-6R to form a solubilized IL-6/sIL-6R complex which is able to bind to gp130 alone. Gp130 is ubiquitously expressed on all cell membranes in the body, enabling an expansive response to IL-6 in the *trans* pathway [9].

There is a soluble form of gp130 (sgp130), generated by alternative splicing, that has been found to be a specific inhibitor of IL-6 *trans*-signaling [15]. Sgp130 is present in high levels in the blood and binds with 100 times higher affinity to the IL-6/sIL-6R complex than IL-6 binding to sIL-6R alone. As a result sgp130 can act as a buffer to immediately neutralize the IL-6/sIL-6R complex that otherwise initiates *trans* IL-6 signaling by binding to membrane bound gp130 [16]. The soluble form of gp130 (sgp130) can thus regulate *trans* IL-6 signaling, while the transmembrane form of gp130 along with the membrane bound IL-6R initiates the *cis* pathways.

This study sought to evaluate if there would be evidence of increased activity of the pro-inflammatory pathway of IL-6 signaling, the *trans* signaling pathway, in patients who have renal scarring and a history of febrile UTI. In studying the pathogenesis of post-pyelonephritic scarring, studies have highlighted the contribution of recruited inflammatory cells such as neutrophils and macrophages to local tissue damage [5]. Our study did not find differences in the absolute levels of IL-6, sIL-6R, and sgp130 in the urine of children with a history of febrile UTI with or without renal scarring. The only difference we found was the lack of correlation between IL-6 levels and sgp130 in those with renal scarring. This finding is intriguing as sgp130 levels otherwise correlated with sIL-6R values in both groups. This might suggest that sgp130 normally compensates for rising levels of sIL-6R to thus inhibit *trans* signaling. This can be reflected in the direct relationship between these values as demonstrated in our graphs in Figure 2. Yet, while levels of sgp130 also directly correlate with IL-6 levels in those without renal scarring, there is no such relationship in those with scarring, potentially suggesting some form of dysregulation of the *trans* IL-6 signaling pathway that might help distinguish the potential difference in outcome between these groups with a history of febrile UTI. This led us to wonder if the ratio of these markers might be important; however, upon analysis, these were not significantly different between groups.

Prior work has found that IL-6 expression alone is associated with the development of renal scarring. Sheu et al found that serum and urine IL-6 levels in children at the time of acute pyelonephritis were significantly higher in children who subsequently developed renal scarring versus those who did not on DMSA six months later [17]. Similarly, Rodriguez et al found that serum IL-6 levels were significantly higher at the time of UTI in those who later developed renal scarring [18].

Our study is novel in that it is the first to report sgp130 expression in the urine in UTI and to consider the correlation and ratio of these markers to each other. Prior studies have explored the expression of sIL-6R in the urine and serum of patients with UTI but have not looked at its expression relative to IL-6 levels. Tullus et al found that sIL-6R in the urine of healthy children was significantly higher than in children with recurrent pyelonephritis, fever unrelated to pyelonephritis, and children 6 weeks after pyelonephritis. These findings suggest utilization of sIL-6R reserves in patients over the time of their infection and in those with repeated infection, likely as a result of IL-6 induction in both scenarios, with resulting lowering of available sIL-6R. Similar to our findings, they did not find a difference in sIL-6R levels between children who had had an UTI who developed renal scarring or not on DMSA at 1 year of follow-up [19]. Jacobson et al found that while serum sIL-6R levels were not significantly different between children immediately at time of acute pyelonephritis as compared to healthy controls, serum sIL-6R was significantly higher two weeks after UTI with antibiotic treatment. Furthermore, serum sIL-6R levels two weeks after UTI were inversely correlated with glomerular filtration rates, suggesting that serum sIL-6R may potentiate the renal consequences of infection [20].

There are multiple limitations of this study. The sample size is small. The groups did differ by race, which does correlate with IL-6 levels, and number of febrile UTIs, which could be a confounder as to the development of renal scarring. We also acknowledge that fever is not

necessarily indicative of renal involvement at time of UTI; and that we did not have consistent imaging at the time of UTI to show renal involvement that could predispose to developing renal scarring. We also cannot definitively conclude that the scarring seen was a result of their prior UTI given we did not necessarily have baseline imaging prior to infection on most patients to know if the post-UTI imaging findings were new. We also did not have DMSA scans on all patients, particularly those in the NS group, to be able to absolutely confirm the lack of scarring. As a result, we were willing to accept RUS diagnosis of renal scarring, which is not the gold standard for diagnosing scarring. It is interesting to note, however, that 16 of the 17 patients with a DMSA had similar findings of scarring on their RUS as their DMSA, even with matching laterality. In addition, we included patients with VUR in our UTI cohorts which limited our ability to conclude scarring was a direct result of *trans* IL-6 signaling independent of VUR. While we recognize these concerns, this study serves as a pilot evaluation of the *trans* IL-6 signaling pathway in UTI. Ideally, future studies would evaluate markers of *trans* IL-6 signaling at the time of infection to determine the impact of this pathway in the acute setting and even consist of serial urine samples after recovery in order to determine if it might be the change in expression not just the absolute number that is significant. Understanding drivers of UTI pathology would enable their targeting and potentially alter UTI susceptibility or outcome.

Conclusions

Our study did not find differences in the absolute levels of IL-6, sIL-6R, and sgp130 in the urine of children with a history of febrile UTI with or without renal scarring

Acknowledgments

Funding sources: American Association of Pediatric Urologists (C.B.C.), U.S. National Institutes of Health grants K08-DK122119 (C.B.C.), K08-DK102594 (B.B.). B.B. is also supported by a Norman Siegel Research Scholar Grant from the ASN Foundation for Kidney Research.

Abbreviations:

DMSA	dimercaptosuccinic acid
ELISA	enzyme-linked immunosorbent assays
IL-6	interleukin-6
IRB	institutional review board
NS	non-scarred
RUS	renal ultrasound
S	scarred
sIL-6R	soluble interleukin-6 receptor
sgp130	soluble gp130
UCr	urine creatinine

UTI	urinary tract infection
VCUG	voiding cystourethrogram
VUR	vesicoureteral reflux

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Figure 1:

ELISA results of markers of *trans* IL-6 signaling in the urine of patients with a history of febrile UTI who do not have renal scarring (NS) as compared to those who do (S). No results were significantly different.

Figure 2:

Comparative studies of markers of *trans* IL-6 signaling. Scatter plots with line of best fit for those significantly correlated in **A)** non-scarred and **B)** scarred patients. **C)** Ratio of markers of *trans* IL-6 signaling in the urine of patients with a history of febrile UTI without renal scarring (NS) as compared to those with scarring (S). No results were significantly different.

Table 1.
Patient demographics.

Patient and corresponding imaging information for patients with a history of UTI and healthy controls (HC).

	No Renal Scarring (n=27)	Renal Scarring (n=23)	p-value
Mean age in years (median; range)	6.5 (4.7; 0.6-18.7)	7.6 (7.8; 2.2-19.9)	0.2204
Sex			0.8340
- Female (%)	24 (89)	20 (87)	
-Male (%)	3 (11)	3 (13)	
Race			0.0141
- Caucasian	25	14	
- AA	0	3	
- Hispanic	2	2	
- SE Asian	0	2	
- Biracial	0	2	
Circumcision status			
- Circumcised (%)	2 (67)	3 (100)	>0.9999
Method of urine collection			0.7582
- Free void (%)	18 (67)	17 (74)	
- Catheterization (%)	9 (33)	6 (26)	
Mean number of febrile UTIs (median; range)	1.5 (1.0; 1-3)	2.1 (2; 1-4)	0.0057
Mean months from UTI (median; range)	11.0 (2.8; 0.3-92.1)	14.6 (5.7; 1.2-69.4)	0.0710
- Individuals with unknown time from UTI	2	4	
Individuals with imaging at time of UTI	7	7	0.7610
- Imaging confirming renal involvement	3	3	>0.9999
Imaging			
- RUS	27	23	>0.9999
- Scarring	0	23	
- Hydro	5 (SFU grade 1)	4 (SFU grade 1/2)	
- Duplex	3	2	
- Cyst	1	0	
- VCUG	21	22	0.1068
- VUR present	16	20	0.2404
- dilating VUR	11	18	0.2036
- DMSA	0	15	<0.0001

UTI: urinary tract infection

RUS: renal ultrasound

VCUG: voiding cystourethrogram

VUR: vesicoureteral reflux

DMSA: dimercaptosuccinic acid