

FOXP3⁺ T cells are present in kidney biopsy samples in children with tubulointerstitial nephritis and uveitis syndrome

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Abstract

Background Tubulointerstitial nephritis (TIN) is an inflammatory disease with unknown pathogenesis. In order to evaluate a possible role of regulatory T cells (Treg) in the pathophysiology of TIN with (TINU) and without uveitis, we investigated the presence and quantity of FOXP3⁺ T regulatory lymphocytes in diagnostic kidney biopsies from pediatric patients.

Methods A total of 33 patients (14 TIN and 19 TINU) were enrolled. The quantity of CD4⁺, FOXP3⁺ and double-positive T cells in formalin-fixed kidney biopsies was determined using double label immunohistochemistry with anti-human CD4 and FOXP3 antibodies.

Results FOXP3 staining was successful in all 33 patients. In patients with chronic uveitis the density of FOXP3⁺ cells was significantly lower ($p=0.046$) than in TIN patients without uveitis or with uveitis lasting < 3 months. CD4⁺ staining was successful in 23 patients. The density of all lymphocytes (CD4⁺, CD4⁺FOXP3⁺ and FOXP3⁺ cells) was significantly lower ($p=0.023$) in patients with chronic uveitis when compared to other patients.

Conclusions FOXP3⁺ T cells are present in kidney biopsy samples from TIN and TINU patients. In patients with chronic uveitis the density of FOXP3⁺ T cells is significantly lower than in other patients, suggesting a different pathomechanism for these clinical conditions.

Keywords: TIN, regulatory T cell, child, biopsy, uveitis, FOXP3

Introduction

Tubulointerstitial nephritis (TIN) is an inflammatory process affecting primarily renal interstitium and tubular wall without significant glomerular or vascular involvement [1]. In some cases renal symptoms are accompanied by uveal inflammation (TINU-syndrome) [2]. TIN has been reported to cause approximately 7% of acute kidney injuries (AKI) in pediatric population [3]. In adult population, TIN accounts approximately for 1.5–11% of all diagnostic renal biopsies [4].

TIN may be caused by various viruses and bacterial infections as well as by numerous drugs [5]. However, especially in children and adolescents the pathomechanism of TIN remains unknown. The diagnosis of TIN is based on histological findings in renal biopsy. Typically, there are T-cell infiltrations, plasma cells, and eosinophils in the renal interstitium and tubular walls [6]. Immunofluorescence usually remains negative, suggesting that TIN is not an antibody-mediated disease. Both TIN and TINU syndrome have been shown to be enriched in patients with certain HLA type, potentially reflecting underlying autoimmune mechanisms [7-9].

Naturally occurring CD4⁺CD25⁺ regulatory T cells (nTreg) actively suppress pathological and physiological immune responses. These cells contribute to the maintenance of immunological self-tolerance and immune homeostasis by inhibiting the activation, proliferation and function of other lymphocytes in physiological or pathological immune responses [10]. Tregs constantly express the x-linked transcription factor forkhead box protein 3 (FOXP3) which is the major regulator the development, homeostasis and function of regulatory T lymphocytes [11].

Tregs have been shown to be important in many different immunological conditions such as renal allograft rejection [12, 13] as well as in many autoimmune diseases such as systemic lupus erythematosus (SLE), multiple sclerosis (MS), ankylosing spondylitis (AS) [14] and in glomerulonephritis (GN) [15, 16]. Mutations in FOXP3 gene lead to dysfunctional Treg cells causing immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) which accompanies severe autoimmune diseases e.g. diabetes, inflammatory bowel disease, allergy and different forms of renal injury [17].

The data regarding the expression of FOXP3⁺ T helper cells in renal biopsy samples is restricted to few study reports [18, 19]. In the present study, we aimed to evaluate the presence of these cells in the kidney biopsy samples of pediatric TIN patients. We also studied whether the expression of FOXP3⁺ T cells differ between the patients with TIN and TINU patients with or without chronic uveitis.

Materials and Methods

In this nationwide study, all pediatric patients (age <16 years of age) diagnosed with biopsy-proven idiopathic TIN between 2001-2014 were enrolled. All except six patients had participated in our previous studies. All TIN diagnoses were based on kidney biopsies, which were performed before any treatment was given. All patients had meticulous work-up to exclude possible underlying conditions, such as respiratory infections, sarcoidosis, connective tissue disorder, lymphoma. Also patients with suspected self-limiting, drug-induced TIN were excluded. [2, 20, 21]. A total of 42 patients from five university hospitals in Finland were found, and stored biopsy material was available from 33 patients. Nineteen patients (58%) had uveitis (TINU syndrome) and 14 (42%) of them had chronic uveitis. The median age at the time of diagnosis was 12.7 (9.4-14.4) years (Table 1). In ten (30%) cases immunohistochemical double-staining for both CD4⁺ and FOXP3⁺ could not be done due to insufficient biopsy material, disorientation of the sample on the slide or poor quality of the staining. Consequently, double staining of both CD4⁺ and FOXP3⁺ cells was successful in 23 patients.

All patients were followed up by pediatric nephrologist and ophthalmologist at least for one year after the diagnosis of TIN. Uveitis was classified according to standardization of uveitis nomenclature (SUN) criteria. Chronic uveitis was defined as uveitis that had lasted more than three months despite of treatment and/or with relapse within three months after discontinuing treatment.

Laboratory samples were taken at the time of the diagnostic kidney biopsy. Plasma creatinine (P-Crea), C-reactive protein (CRP), blood erythrocyte sedimentation rate (ESR), and urinary excretion of low molecular weight (LMW) proteinuria by measuring β -2-microglobulin (β -2-MG) were analysed from each patient. Glomerular filtration rate (GFR) was measured either by Cr-EDTA or iohexol clearance. Estimated GFR was calculated using bedside Schwartz formula.

Immunohistochemistry

Kidney biopsies were routinely formalin-fixed and embedded in paraffin blocks after biopsy. As part of this study, paraffin blocks were cut to 2,5- μ m sections. The second phase was antigen retrieval with Tris-EDTA (pH 9) in microwave. The quantity of CD4⁺, FOXP3⁺ and CD4⁺FOXP3⁺ double-positive T cells were determined with double-label immunohistochemistry using anti-human CD4 (NovacastraTM Product Code NCL-L-CD4-368 with 1:50 dilution) and FOXP3 (Abcam, 236A/E7 with 1:100 dilution) antibodies. Immunostainings were performed using Dako REALTM EnVisionTM Detection System (Dako code K5007). For CD4⁺ cells DAB-peroxidase substrate was used from the same kit. For FOXP3 Vector SG substrate kit (catalog number SK-4700 by Vector laboratories) was used. (Figure 1)

The areal density of positive cells was determined with high magnification (x 40 objective) and a graticule corresponding field size of 0.063 mm². Cells were counted in 5 random fields and the mean value was calculated. The quality of the CD4⁺ and FOXP3⁺ stainings was confirmed using positive control sample from tonsillar tissue. In addition to the kidney biopsy samples from the 33 TIN patients, 25 kidney biopsy samples from Henoch-Schönlein nephritis (HSP) patients were stained as control samples (Figure 1).

Statistical analysis

We used IBM SPSS Statistics version 22 for statistical analysis. Due to skewed distribution of each measured continuous variable we used Mann-Whitney U test to compare the results between TIN and TINU patients with or without chronic uveitis. A two tailed p-value <0.05 was considered to indicate statistical significance.

Results

General findings

The demographic data of the patients is presented in Table 1. In TIN patients, ESR and P-Crea were significantly higher ($p=0.010$ and $p=0.012$) and GFR was significantly lower ($p=0.014$) than in TINU patients at the time of diagnosis. Furthermore, in patients with chronic uveitis ESR was significantly higher ($p=0.039$) than in patients without chronic uveitis (Table 1).

Immunohistochemistry

Immunohistochemical staining for FOXP3 was successful in all 33 patients. The cell densities are presented in Table 2 separately for TIN patients, for patients with TINU, for patients with TINU and chronic uveitis, and for patients with TINU without chronic uveitis. In patients with chronic uveitis the density of FOXP3⁺ T cells was significantly lower ($p=0.046$) than in TIN patients without uveitis or uveitis lasting <3 months (Table 2 and Figure 2).

CD4⁺ staining was successful in 23 (70%) patients. The age and gender distribution of these 23 patients did not differ significantly from the total study population. The prevalence of uveitis (52%, $n=12$) and chronic uveitis (35%, $n=8$) were also comparable to the original population (data not shown). The results from the double staining experiments are shown in Table 2 and Figure 2. The overall density of lymphocytes was significantly lower ($p=0.023$) in patients with chronic uveitis compared to other patients.

Immunohistochemical stainings performed to 25 kidney biopsy samples from HSP patients were all successful. In all 25 HSP samples the density of CD4⁺ and FOXP3⁺ and double positive cells were almost nonexistent (Figure 1).

Discussion

TIN/TINU syndrome is a rare condition with various etiology but of unknown immunopathology [22]. In the present study, we have demonstrated for the first time that FOXP3⁺ T cells are present in kidney biopsy samples from pediatric TIN patients. We also showed some evidence for a significantly lower density of CD4⁺ and/or FOXP3⁺ T cells in TINU patients with chronic uveitis compared to other patients. It may well be that the immune response in patients with chronic TINU is different from other patients leading to prolonged inflammatory process.

Renal biopsies of TIN patients typically show presence of T lymphocytes and other mononuclear cells and occasionally eosinophils [6]. The pathomechanism in TIN is therefore suggested to be antigen-driven molecular mimicking leading to autoimmune type disease [23]. A recent study by Cheng et al. has shown evidence about increased number of dendritic cells in kidney biopsy samples from TIN patients and from patients with lupus nephritis (LN) when compared to samples from minimal change disease patients [24]. In the present study, we found CD4⁺FOXP3⁺ T cells, i.e. potential Tregs, in the biopsy samples from TIN patients. Interestingly, in patients with chronic uveitis indicating persistent autoimmune response, the density of these cells was lower than in patients with other forms of TIN. It may well be that in these patients Treg activation is for some reason dampened, which leads to prolonged inflammatory response and chronic form of the disease. For comparison, the density of CD4⁺ and FOXP3⁺ cells were almost non-existing in our biopsy samples from HSP patients what is in line with previous studies [25, 26].

No previous data regarding Tregs in TINU patients has been published. However, it has been shown that the quantity of FOXP3⁺ T cells in kidney transplants positively correlates with long-term graft function. Patients with no chronic damage in biopsy had significantly higher percentage of CD3⁺/FOXP3⁺T cells compared to patients with any chronic histologic damage [12, 13]. Moreover, it has been shown that the amount of FOPX3⁺ T cells within the entire CD4⁺ T cell population is significantly higher in patients with subclinical or mild rejection than in patients with acute rejection and it correlates negatively with graft function at the time of biopsy [27], suggesting that Tregs may reduce interstitial inflammation. Polymorphisms in the FOXP3 gene and in genes coding various cytokines has been shown to cause altered inflammatory response, which may result in autoimmune disease [28].

The potential role of Tregs in other autoimmune kidney diseases has been studied in both murine and human models. In anti-neutrophil cytoplasmic antibody (ANCA) -associated vasculitis (AAV), the findings from

human studies are conflicting regarding the amount of circulating Tregs but there is evidence that FOXP3⁺ Tregs are functionally impaired, with diminished capability to suppress effector T cell (Teff) proliferation [29-34]. There are several reports about reduced quantity and function of Treg cells in SLE patients. Xing et al. demonstrated decreased frequency of CD4⁺CD25⁺FOXP3⁺ Treg cells in peripheral blood with lupus nephritis (LN) compared to SLE patients without nephritis [35]. In the study by Afeltra et al. the ratio of FOXP3⁺/CD3⁺ cells was significantly lower in lupus nephritis class IV compared to lupus nephritis class V, to patients with nephroangiosclerosis and to 6 patients with acute TIN used as control patients [19].

The major limitation of the present study is relatively low number of patients. However, TIN is a rare disease and this is by far one of the largest studies available in the literature. In addition, all patients were studied and followed up by pediatric nephrologist and ophthalmologist using the same protocol [21]. This meticulous work-up guarantees that the study population represents patients with idiopathic TIN or TINU with or without chronic uveitis. In addition, based on our results we are not able to draw any conclusions about the mechanism of uveitis in this patient cohort. However, several other autoimmune diseases that are accompanied with uveitis e.g. ankylosing spondylitis [36], juvenile idiopathic arthritis [37] and SLE [38] have been shown to have impaired Treg production or function.

Conclusions

To the best of our knowledge this is the first report comparing the presence of FOXP3⁺ T cells in biopsy samples from patients with TINU and patients with TINU and chronic uveitis. FOXP3⁺ T cells could be found in all patients; however, in patients with chronic uveitis the density of Tregs was significantly lower than in other patients. This may indicate a different pathomechanism behind these clinical conditions, implying that in TINU patients with chronic uveitis the pathomechanism may be of autoimmune origin. Due to the relatively low number of patients replication studies with larger patient cohorts are needed.

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Compliance with Ethical Standards

Conflict of Interest

The authors declare that they have no conflict of interest.

Statement of human rights

The study adhered to the tenets of the declaration of Helsinki and the study protocol was approved by ethics committee.

All participants and / or their parents gave their informed consent for the study.

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Fig. 1 Immunohistochemical staining of CD4⁺ and FOXP3⁺. **a** Renal biopsy from a TINU patient with chronic uveitis, 20xmagnification. **b** Same specimen with 40x magnification. **c** Control tonsilla tissue, 40x magnification. **d** Renal biopsy sample from a HSP patient, 40x magnification.

TINU, tubulointerstitial nephritis and uveitis syndrome

* Double positive CD4⁺FOXP3⁺ cell # CD4⁺ cell

Fig. 2 Comparison of FOXP3⁺ and/or CD4⁺ cell density between TIN, TINU and patients with or without chronic uveitis **a** Difference in all FOXP3⁺ cells between TIN and TINU patients, $n= 33$. **b** Difference in all FOXP3⁺ cells between patients with and without chronic uveitis, $n=33$. **c** Difference between all cells (CD4⁺FOXP3⁺, CD4⁺ and FOXP3⁺ cells) between patients with and without chronic uveitis, $n= 23$. TIN, tubulointerstitial nephritis; TINU, tubulointerstitial nephritis and uveitis syndrome; Chronic uveitis was defined as uveitis that had lasted more than three months despite of treatment and/or with relapse with in three months after discontinuing treatment; Each boxplot presents the median and the 25th and 75th centiles; Error bars represent the smallest and the largest values

Table 1 Patient demographics and key laboratory findings at the time of the diagnostic biopsy. The data is presented for all patients and depending on the presence and chronicity of uveitis

	All patients <i>n</i> = 33	TIN <i>n</i> = 14	TINU <i>n</i> = 19	<i>p</i> value	TINU with Chronic uveitis <i>n</i> = 14	No chronic uveitis* <i>n</i> = 19	<i>p</i> value	TINU without chronic uveitis <i>n</i> =5 #
Male gender, n (%)	17 (52)	7 (50)	10 (53)		8 (57)	9 (47)		2 (40)
Age, years	12.7 (9.4-14.4)	11.4 (8.5-12.2)	12.7 (12.1-14.3)	0.358	13.2 (12.1-14.4)	11.8 (8.8-12.3)	0.679	13.2 (10.9-15.0)
CRP, mg/L	14.0 (3.0-60.0)	34.0 (4.0-74.0)	13.0 (3.0-53.0)	0.286	13.0 (3.0-53.0)	23.0 (3.0-60.0)	0.650	8.4 (2.5-48.5)
P-Crea, µmol/L	143.0 (108.0-259.0)	254.0 (102.0-332.0)	128.0 (103.0-169.0)	0.012	127.0 (92.0-205.0)	139.0 (103.0-263.0)	0.186	128.0 (99.0-132.0)
U-β2MG, mg/L	20,0 (3.4-48.0)	14,8 (2.3-20.6)	34,0 (12.6-47.0)	0.220	20,0 (3.4-47.0)	20,6 (3.2-42.0)	0.650	38.0 (11.2- 69.7)
ESR, mm/h	95.0 (39.0-116.0)	104.0 (94.0-113.0)	73.0 (29-106)	0.010	64.0 (25.0-92.0)	105.0 (92.0-114.0)	0.039	95.0 (29.0-110.0)
GFR, ml/min/ 1.73m ²	53.0 (28.0-78.0)	31.0 (22.0-97.0)	61.0 (47.0-80.0)	0.014	62.0 (47.0-91.0)	45.0 (27.0-72.0)	0.077	65.4 (45.5-77.5)

TIN, Tubulointerstitial nephritis; TINU, tubulointerstitial nephritis with uveitis syndrome; CRP, C-reactive protein; P-Crea, plasma creatinine concentration; U-β2MG, urine beta-2-microglobulin concentration; ESR, erythrocyte sedimentation rate; GFR, glomerular filtration rate. Chronic uveitis was defined as uveitis that had lasted more than three months despite of treatment and/or with relapse with in three months after discontinuing treatment. Reported values are median values and 25th and 75th percentiles are in brackets.

* Including patients with TIN and TINU syndrome without chronic uveitis

There were no statistical differences between TINU patients without chronic uveitis compared to other subgroups

Table 2 Lymphocyte densities in all patients, in patients with TIN and TINU syndrome and depending on the presence of and chronicity of uveitis

	All patients <i>n</i> = 33 [#] /23 [°]	TIN <i>n</i> = 14 [#] /11 [°]	TINU <i>n</i> = 19 [#] /12 [°]	<i>p</i> value	TINU with chronic uveitis <i>n</i> = 14 [#] /8 [°]	TINU without chronic uveitis <i>n</i> = 5 [#] /4 [°]	All patients without chronic uveitis <i>n</i> = 19 [#] /15 [°]	<i>p</i> value chronic uveitis vs. no chronic uveitis
All FOXP3 ⁺ / mm ² <i>n</i> = 33	240 (134-448)	283 (131-432)	230 (141-422)	0.76	187 (134-240)	502 (381-512)	381 (211-502)	0.046
All cells /mm ² * <i>n</i> = 23	583 (362-1261)	1181 (446-1280)	520 (362-658)	0.375	464 (282-556)	1192 (583-1974)	1181 (522-1389)	0.023
CD4 ⁺ FOXP3 ⁺ /mm ² <i>n</i> = 23	141 (67-272)	211 (82-296)	104 (62-197)	0.413	93 (62-157)	248 (83-440)	211 (82-350)	0.131
CD4 ⁺ /mm ² <i>n</i> = 23	333 (138-560)	333 (170-654)	312 (139-402)	0.833	312 (123-373)	764 (160-373)	333 (160-890)	0.325

TIN, tubulointerstitial nephritis; TINU, tubulointerstitial nephritis and uveitis syndrome

Chronic uveitis was defined as uveitis that had lasted more than three months despite of treatment and/or with relapse with in three months after discontinuing treatment.

Reported values are median values and 25th and 75th percentiles are in brackets

* CD4⁺, CD4⁺FOXP3⁺ and FOXP3⁺ cells

All patients with successful FOXP3⁺ staining

° All patients with successful double staining