High levels of circulating interleukin-10 in diabetic nephropathy patients

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ABSTRACT. *Aims.* **The aim of our study was to analyse the level of circulating interleukin-10 (IL-10) and relate it to the grade of albuminuria in patients with diabetic nephropathy (DN) due to type 1 diabetes mellitus (DM). Since IL-10 has met the criteria for an anti-inflammatory and an immunosuppressive cytokine, its activity may be important for clinical outcome of DN.** *Methods.* **The IL-10 level was measured by ELISA in serum samples from thirty patients with DN due to type 1 DM, and compared with thirty patients with type 1 DM without DN and a control group of thirty, healthy, age- and sex-matched people.** *Results.* **We observed a greatly elevated concentration of circulating IL-10 in 30/30 DM patients with DN (mean 140 pg/mL ± 102), compared to DM patients without DN in whom IL-10 was detectable in only 11/30 patients (0.79 pg/mL ± 1.24), and the group of healthy people in whom IL-10 was detectable in only 3/30 donors (0.92 pg/mL ± 0.17). IL-10 appeared to be the strongest independent predictor of albuminuria, followed by HbA_1c, diastolic blood pressure and DN duration. There was a positive correlation between the values of IL-10 and albuminuria in DM patients with DN. The patients in the fourth quartile of albuminuria had a distinctly higher concentration of IL-10 than those in the lower quartiles.** *Conclusions.* **The increased concentration of IL-10 in the serum samples from DM patients with DN seems to depend on the severity of the nephropathy. The excessive IL-10 production may indirectly contribute towards DN progression. On the other hand, it may explain the relatively long course of diabetic nephropathy.**

Keywords: interleukin-10, type 1 diabetes mellitus, nephropathy

Insulin-dependent type 1 diabetes mellitus (type 1 DM) confers an increased risk of various viral, bacterial and fungal infections [1-3]. Moreover, diabetic nephropathy (DN) in haemodialysis patients is an independent predictor of mortality [4]. Although, uncontrolled glycaemia has commonly been regarded as a causative factor of increased morbidity and mortality in these patients, this does not represent the whole picture. The concomitance between the long course of the disease and the susceptibility to infection leads to the suggestion that anti-inflammatory and immune-suppressive factors play an essential role in the pathogenesis of DN. These factors may slow down the course of diabetic nephropathy through a reduction of the inflammatory processes, while simultaneously rendering the patient more susceptible to infection. Interleukin-10 (IL-10) fulfils the criteria for an anti-inflammatory and immunosuppressive cytokine.

IL-10 has been shown to limit the cascade of pro-inflammatory cytokines activation [5] and to down-regulate T cell-mediated immune responses [6]. In accordance with its anti-inflammatory activity, high concentrations of IL-10 in critically ill, septic patients protected them from death [7], while low levels were found in non-survivors [8].

The TH2 cytokines have been documented to as dominating in nephropathies with a relatively long pre-dialysis or pre-transplantation course such as idiopathic membranous nephropathy [9], primary IgA nephropathy [10] and HBV-associated membranous disease [11], all of which are characterised by an increased production of IL-10 in different *in vitro* experimental settings. The role of the TH1 system in nephropathies is the reverse, as a high expression of TH1 cytokine transcripts predicts severe glomerular lesions and poor clinical outcome [12].

The studies on experimentally induced, passive, antiglomerular basement membrane antibody-induced model of glomerulonephritis in rats showed that systemic treatment with IL-10 significantly reduced the degree of proteinuria and systemic inflammation, and attenuated renal injury [13]. Those rats with mesangial, proliferative glomerulonephritis that received treatment with IL-10, displayed a lesser degree of histological lesions, limited cellular proliferation and a lower expression of inflammatory mediators [14]. When IL-10 was delivered by means of gene therapy, to mice with naturally occurring renal failure, it effectively reduced the level of proteinuria and progression to glomerulosclerosis [15].

DN has been regarded as a slowly evolving disease with a long pre-dialysis period, which suggests that the course of the disease may be shaped by IL-10.

We have not encountered any reports analysing interleukin-10 in DN patients. The fragmentary knowledge of the different nephropathies and the absence of data focusing on diabetic nephropathy, inspired us to analyse the level of IL-10 in the serum from patients with DN due to long standing type 1 DM.

PATIENTS AND METHODS

Thirty patients (13 females and 17 males, age 41.76 ± 11.14 years) with diabetic nephropathy and type 1 DM, were recruited from The Regional Diabetic Centre at the Medical University in Gdańsk. Type 1 DM was distinguished from type 2 diabetes by the presence of low BMI, in combination with hyperglycaemia, ketonuria and a short duration of symptoms such as polyuria, polydipsia, and weight loss. Their metabolic state was regularly established by an examination of the fasting glucose level and glycosylated haemoglobin level (HbA_1c). Moreover, Hb, creatinine, creatinine clearance, total cholesterol, HDL-cholesterol and triglycerides were monitored. HbA_1c was measured using an immunoturbidometric method involving a « Unimate 3 » set (Hoffmann-La Roche AG, Germany) with a normal range of values of 3.0-6.0%. Fasting serum levels of total cholesterol, HDL-cholesterol and triglycerides were measured with the enzymatic kits: « Comray Chol », « Comray HDL-Direct » and « Comray-TG » (P.Z. Comray, Poland). Systolic and diastolic blood pressures were measured using an automatic device, with the patient in the supine position after 5 min rest. The mean of all of the blood pressure recordings measured in each patient during the six months prior to the study was used. Mean systolic blood pressure below 140 mmHg and mean diastolic below 90 mmHg were set as threshold values for normal blood pressure. The urinary albumin excretion rate was measured in three, timed, overnight urine samples at least twice a year using an immuno-turbidometric assay involving a « Tina-quant® » (Boehringer Mannheim GmbH, Germany) kit. Urinary albumin excretion was expressed as the mean of all 24h collections obtained during the six months prior to our examination. The serum creatinine level was measured using the « CREA » assay system (Boehringer Mannheim GmbH, Germany) with the reference values as follows: for men (< 50 years) < 115 µmol/L and women < 97 µmol/L. All patients presented with overt DN, which was characterised as an albumin excretion rate above 300 mg/24h (macroalbuminuria), in at least two out of three urine samples during the year, in the absence of evidence of any other kidney or urinary tract disease. All patients were receiving angiotensin-converting enzyme (ACE) inhibitors, four of them in combination with diuretics. Twenty four patients received HMG-CoA reductase inhibitors. The patients were not receiving steroids or non-steroidal anti-inflammatory drugs. The DN patients required insulin treatment at a dose of 20-66 U per day. They had not suffered from any acute or chronic diseases for three months prior to the examination. The second group of 30, type 1 DM patients without diabetic nephropathy (17 females and 13 males, age 37.12 ± 13.30 years) were also recruited from the Regional Diabetic Centre at the Medical

University in Gdańsk. Their metabolic state was established similarly to that of the group with DN. All patients were receiving angiotensin-converting enzyme (ACE) inhibitors. Ten patients received HMG-CoA reductase inhibitors. They did not receive steroids or non-steroidal anti-inflammatory drugs. The patients required insulin treatment at a dose of 20-60 U per day. They had not suffered from any acute or chronic diseases for three month prior to the examination. In both groups, patients with cardiovascular disease had been excluded from the study. The control group consisted of 30 healthy subjects (13 females and 17 males, age 42.5 ± 8.2 years) who did not suffer from any acute or chronic disease and who had not received drugs affecting immune system. Written, informed consent was obtained from all patients and the healthy controls. This study was approved by the Ethics Committee of The Medical University of Gdańsk (TKEBN/522/02) and the investigation was carried out in accordance with the principles of the Declaration of Helsinki as revised in 1996.

Blood collection

Blood samples were collected between 8.00 and 9.00 after an overnight fast. The serum was separated from the venous blood within 30 min and kept frozen at -80 °C, up to three months prior to analysis. All determinations were done on the same sample of blood.

Determination of IL-10

Medgenix solid phase ELISA kits (Biosource, Belgium) were used for the determination of IL-10. The ultra-sensitive assay was applied (range 0.78-50 pg/mL) to the samples from the DM patients, and the less sensitive test was used (11-1335 pg/mL) for the samples from the DN patients. The serum samples were incubated on microtitre plates coated with capture monoclonal, anti-IL-10 antibodies. Next, the anti-IL-10 HRP conjugate was added, followed by chromogenic solution TMB. The absorbance was read at 650 nm on the automated plate reader (Multiscan MCC/340, Labsystems, Helsinki, Finland). The reference curve was prepared according to the manufacturer's recommendations. No significant cross-reaction was observed between IL-10 and: IL-1, IL-2, IL-3, IL-5, IL-6, TNF-α, TNF-β, IFN-α, IFN-β, GM-CSF, OSM, MIP-1α, MIF, MCP-1, G-CSF and RANTES.

Statistical analysis

The results were analysed using the Statistica, Version 6 program (StatSoft, Pl). The Shapiro-Wilk's test was used to evaluate the normality of variables. The differences between the groups of normally distributed variables were calculated with the unpaired Student's *t* test; with the results presented as arithmetic means ± SD. For comparison of the skew-distributed variables, the non-parametric Mann-Whitney U test was applied. The results of the Mann-Whitney U test are presented as medians (25th and 75th percentiles). The differences in concentration of IL-10 between patients representing the albuminuria quartiles were calculated with the ANOVA Kruskal-Wallis test. The Spearman correlation coefficient was calculated to determine any correlation between the concentration of IL-10 and albuminuria. The multivariate linear stepwise regres-

sion was applied to assess independent predictors of albuminuria. The non-normally distributed values were log-transformed before multivariate regression was performed. The level of significance was set at $p < 0.05$ and two-sided tests were performed as the standard.

RESULTS

Basic clinical characteristics and parameters related to neuropathy

The parameters related to nephropathy, and the basic clinical characteristics are presented in *tables 1* and *2*.

The group of 30 patients developed overt nephropathy due to the long-standing, type 1 DM. They were characterised by albuminuria in the range of 300 and 3,600 mg/24h (mean: 2064 ± 1030), a creatinine level of 110.5 μmol/L (88.4-150.28) and a creatinine clearance of 1.59 mL/s (1.49-1.66). They displayed elevated levels of fasting glucose, HbA$_1$c, and they required insulin treatment. Systolic and diastolic blood pressures were within the range of the reference values due to the medical treatment.

The group of 30, non-DN patients with Type 1 DM were characterised by an albumin excretion rate < 30 mg/24h, a creatinine level of 102.5 μmol/L (94.5-109.6) and creati-

nine clearance ≥ 1.0 mL/s. This group had elevated levels of fasting glucose, HbA$_1$c, and required insulin treatment. Their systolic and diastolic blood pressures, controlled by the medical treatment, were also within the range of the reference values (*tables 1* and *2*).

The basic clinical characteristics of DN patients were similar to that of Type 1 DM patients. The differences between values for diabetes duration, BMI, fasting glucose, HbA$_1$c, Hb, triglycerides, total cholesterol, HDL-cholesterol and insulin doses were not statistically significant (*table 2*).

Circulating levels of IL-10

The concentration of circulating IL-10 was greatly elevated (10 to 100 times) in 30/30 DM patients with DN (mean 140 pg/mL ± 102), as compared to DM patients without DN, in whom IL-10 was low, and detectable in only 11/30 patients (0.79 pg/mL ± 1.24). These low IL-10 serum concentrations in DM patients without DN were comparable with values in a group of healthy donors in whom IL-10 was low, and detectable in only 3/30 donors (0.92 pg/mL ± 0.17).

The differences between the three groups (p = 0.04), as well as between DM patients with DN and DM patients

Table 1
Parameters related to the nephropathy

Parameter	DN patients	DM patients	Healthy controls
Number of patients	30	30	30
Patients with hypertension	25	20	0
Systolic blood pressure (mmHg)	135 (125-140)	125 (117–130)	120 (115–125)
Diastolic blood pressure (mmHg)	80 (80-85)	80 (75-80)	79 (70-80)
Creatinine (μmol/L)	110.5 (88.4-150.28)	102.5 (94.5-109.6)	-
Creatinine clearance (mL/s)	1.59 (1.49-1.66)	≥ 1.0	-
Albumin excretion rate (mg/24h) [a]	2064 ± 1030	< 30	-

The values are presented as medians (25th and 75th percentiles). DN patients = patients with diabetic nephropathy due to type 1 diabetes mellitus. DM patients = patients with type 1 diabetes mellitus without diabetic nephropathy.
[a] The values are presented as arithmetic means ± SD.

Table 2
Basic clinical parameters

Parameter	DN patients	DM patients	Healthy controls
Age (years) [a]	41.76 ± 11.14	37.12 ±13.30	42.5 ± 8.20
Duration of the diabetes (years) [a]	22.76 ± 7.30	20.66 ± 9.21	-
Duration of the DN (years) [a]	7.50 ± 2.68	-	-
Smokers (n,%)	6 (20%)	5 (17%)	7 (23%)
Body mass index (kg/m^2) [a]	24.56 ± 3.00	23.44 ± 1.22	24.70 ± 2.90
Fasting glucose (mmol/L) [a]	8.58 ± 1.75	8.65 ± 1.75	5.20 ± 0.70
HbA$_1$c (%)	7.95 (7.6-8.4)	8.2 (6.6-8.8)	-
Hb (g/dL)	13.9 (13.6-14.3)	12.08 (10.5-12.0)	13.76 (13.5-14.6)
Triglycerides (mmol/L) [a]	1.40 ± 0.66	1.23 ± 0.52	1.23 ± 0.52
Total cholesterol (mmol/L)	5.34 (4.82-5.88)	5.34 (4.87-.16)	5.1 (4.5-5.7)
Cholesterol-HDL (mmol/L)	1.49 (1.29-1.83)	1.75 (1.47-.16)	1.5 (1.33-1.88)
Dose of insulin (U/day)	46 (33-56)	38,5 (30-44)	-
n	n = 30	n = 30	n = 30

The values are presented as medians (25th and 75th percentiles). DN patients = patients with diabetic nephropathy due to type 1 diabetes mellitus. DM patients = patients with type 1 diabetes mellitus without diabetic nephropathy.
[a] The values are presented as arithmetic means ± SD.

Table 3
Circulating levels of IL-10

IL-10 pg/mL	DN patients	DM patients	Healthy controls
[a]	121.0 (70-180)	0.00 (0.0-1.5)	0.92 ± 0.17
	140.0 ± 102	0.79 ± 1.24	
Statistical significance Mann-Whitney U test		0.0002	0.0001
Number of patients with detectable IL-10 in serum	30/30	11/30	3/30

The values are presented as medians (25th and 75th percentiles). DN patients = patients with diabetic nephropathy due to type 1 diabetes mellitus. DM patients = patients with type 1 diabetes mellitus without diabetic nephropathy.
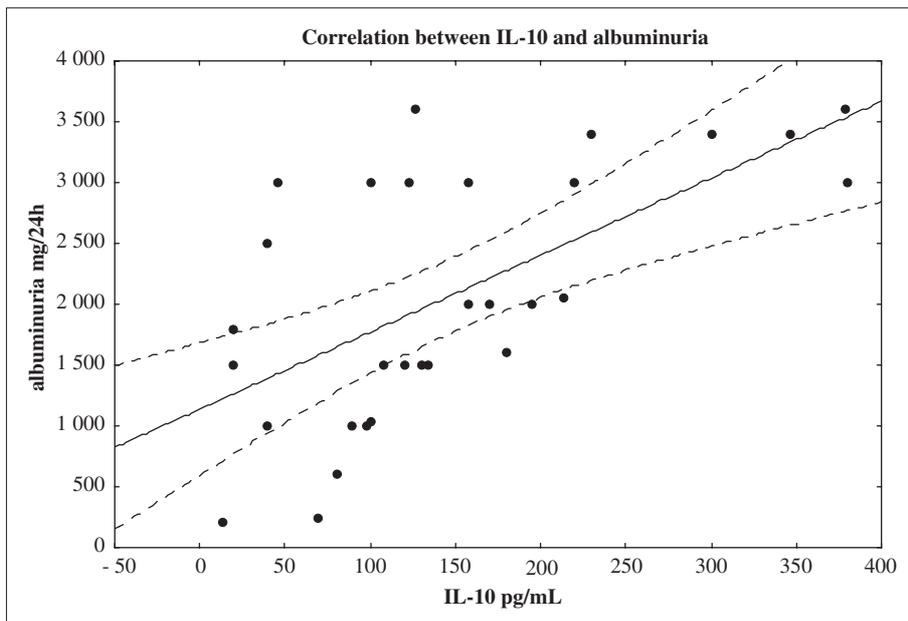[a] The values are presented as arithmetic means ± SD.

Figure 1
The correlation between concentration of IL-10 and albuminuria (Spearman test; r = 0.541; p = 0.002).

without DN (p = 0.0002), and between DM patients with DN and the control (p = 0.0001) group were statistically significant *(table 3)*.

The multivariate stepwise linear regression analysis revealed that IL-10 was the strongest independent predictor of albuminuria (β = 1.700; p = 0.0001) in DM patients with DN. Several other variables also positively influenced the degree of albuminuria, but with less power. They were: HbA$_1$c (β = 0.481; p = 0.00001), diastolic blood pressure (β = 0.281; p = 0.005) and DN duration (β = 0.238; p = 0.03). The multivariate regression test was highly significant (F = 10.81; p = 0.000001) and the model explained 65% of the variance in the albumin excretion rate. In the univariate analysis, albuminuria correlated significantly with the concentration of IL-10 (r = 0.541; p = 0.002) *(figure 1)*.

Patients in the fourth quartile of albuminuria had a decidedly higher concentration of IL-10 than those in the quartiles 1 to 3 (F = 4.6; p = 0.013) *(figure 2)*.

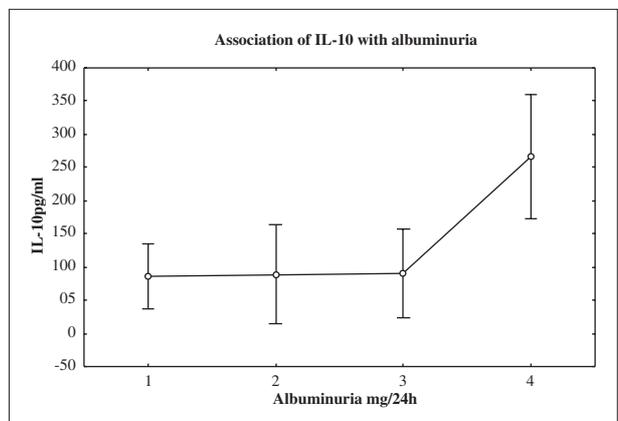No correlations between albuminuria and metabolic parameters were found.

Figure 2
The concentration of IL-10 in relation to the albuminuria quartiles (ANOVA Kruskal-Wallis test; p = 0.013). The values are expressed as medians with the 25th and 75th percentiles. Values of albuminuria (mg/24h) in quartiles were: **1** = 300-1500; **2** = 1501-2000; **3** = 2001-3000; **4** = 3001-3600.

Effect of medical treatment on IL-10 concentration

In our study, according to the results of the multivariate regression test, neither insulin nor treatment with angiotensin-converting enzyme (ACE) inhibitors influenced the level of IL-10 in the patients. The patients receiving a combination of ACE inhibitors and diuretics also had similar levels of IL-10 as the patients treated with the ACE inhibitors alone.

DISCUSSION

In the quest for factors that may shape the course of diabetic nephropathy and influence immune response to microorganisms in DN patients, we hypothesized that IL-10 may fulfil these criteria. In line with this assumption, our results have revealed significantly elevated concentrations of IL-10 in the serum of DN patients. These concentrations by far exceeded the values present in other inflammatory diseases such as atopic dermatitis [16] and juvenile chronic arthritis [17], but were lower in comparison with circulating IL-10 levels in septic patients [8]. They were however, similar to those levels found in patients with intractable Grave's disease [18].

To our knowledge, this is the first study focusing on the association between albuminuria and the level of IL-10 in diabetic nephropathy. Therefore, the answer to the intriguing question about the causative or protective role of IL-10 in DN can only be supposed.

IL-10 seems to be able to modulate the natural course of type 1 DM and of DN at different stages. This effect however, is non-specific and similar to IL-10-dependent effects in other nephropathies. Systemic treatment with IL-10 of NOD mice may delay the onset of the disease or ameliorate its course. This may be achieved by a transfer of the favouring regulatory response dendritic cells [19] or by an induction of IL-10-producing regulatory CD4 + CD25 + cells [20]. The two treatment models are believed to act by restoring, in the NOD mice, the defective anti-inflammatory/TH2 immune responses. The decreased *in vitro* production of IL-10 in first degree relatives of type 1 DM patients [21] suggests that faulty anti-inflammatory/TH2 immune reactions may also underlie human diabetic disease. Immune cells from recent-onset type 1 DM patients are able to produce IL-10 *in vitro*, but in general, the immune response, at this stage, is biased towards the TH1 type cytokines [22]. As the disease progresses, IL-10 may still be found in the serum of some patients but at a concentration of only a few pg/mL, and with no relation to the diabetes-associated, immune parameters [23]. These data, together with the results of our study, suggest that IL-10 probably does not contribute to the pathology of uncomplicated DM later than during the pre-diabetic stage.

The immune activation in DN does not seem to be dependent on antigenic stimulation [24], but appears to be a consequence of hyperglycaemia. The advanced glycation end products (AGE), induce inflammatory immune responses that contribute to the growth of the cortical fibroblasts, collagen synthesis and damage to the proximal tubular epithelial cells [25, 26]. Mesangial expansion, the principal glomerular lesion in diabetic nephropathy, has also been found to be attributable to hyperglycaemia-related inflammation [27].

It is remarkable that DN, recognized as a devastating disease, persists for a long time as relatively mild, systemic inflammation [28]. Given this, our patients with long-lasting DN had relatively well preserved renal function. This may be due to the protective role of high concentrations of IL-10.

What is more, the results of our study provide strong evidence that IL-10 may influence the degree of albuminuria. IL-10 was the strongest independent predictor of albuminuria in DN patients. In the univariate analysis, albuminuria significantly correlated with the concentration of IL-10, and patients in the fourth quartile of albuminuria had a markedly higher concentration of IL-10 than those in quartiles 1 to 3. Other variables, such as HbA_1c, diastolic blood pressure and DN duration influenced the degree of albuminuria with less power. The possible mechanism of action of IL-10 may be inferred by analogy to other nephropathies. It has been found that inflammatory/TH1 and anti-inflammatory/TH2 cytokine transcripts are already weakly expressed in the normal, healthy kidney [29], and an enhancement of their expression is noticeable from the very beginning in various nephropathies [12, 30]. Clearly, the highest expression of IL-10 was found in those patients with severe proteinuria and extensive glomerular sclerosis, both in immune-mediated [12, 30] as well as non-immune nephropathies [31]. Thus, a joint induction of IL-10 with a set of counteracting cytokines from the start of the disease is a general phenomenon independent of the pathogenesis of the nephropathy. The positive link between IL-10 and albuminuria presented in this paper complements the above cited studies. It also however, brings into question the positive or deleterious effect of IL-10 on the albumin excretion rate. It may be concluded from an animal model of nephropathy that IL-10 is not a causative factor of DN, but rather a regulatory cytokine, opposing the deleterious effect of inflammatory/TH1 cytokines induced by a poor glyco-metabolic control. Three studies on various experimentally-induced glomerulonephritis have demonstrated that treatment with IL-10, regardless of the route of administration, significantly reduced the degree of proteinuria [13, 15] and systemic inflammation [13, 14], attenuated glomerular damage and slowed down the progression to glomerulosclerosis [13, 15]. These papers argue in favour of the protective role of IL-10 in kidney disorders. The questions remains as to how to reconcile, the protective role of IL-10 with the positive correlation found in this study, between the level of IL-10 and albuminuria, which suggests that IL-10 might be a herald of DN progression?

The pro-progressive role of IL-10 in DN seems to be executed indirectly. The production of IL-10 has been documented to be under the strong control of TGF-β [32], and these two cytokines mutually regulate each others' activity [33]. Thus, TGF-β drives the TH1/TH2 balance toward a TH2 immune response with all the positive consequences cited above. In addition however, TGF-β promotes renal cell hypertrophy and stimulates extracellular matrix accumulation, which are two, known hallmarks of diabetic renal disease [34]. Strong expression of TGF-β transcripts has been detected in kidneys of patients with various nephropathies characterized by marked proteinuria, but who have preserved renal function. This expression was related to the degree of renal damage; the

plasma and urine TGF-β 1 protein levels correlated with the amount of protein excretion [35]. In the long term, the extensive systemic secretion of IL-10 in DN patients may contribute to the progression of DN and may be an indicator of marked albuminuria with fairly well preserved renal function.

To summarise, the DN patients were characterised by a greatly elevated level of circulating IL-10, which may explain the long course of the disease with relatively well preserved renal function. Moreover, the excessive IL-10 production in DN patients may indirectly contribute towards the progression of DN.

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