Metabolism Clinical and Experimental 121 (2021) 154804

Contents lists available at ScienceDirect

Metabolism Clinical and Experimental

journal homepage: www.metabolismjournal.com

The IL-8-CXCR1/2 axis contributes to diabetic kidney disease

Cristian Loretelli [a,1], Francesca Rocchio [a,1], Francesca D'Addio [a,1], Moufida Ben Nasr [a,b], Eduardo Castillo-Leon [b], Sergio Dellepiane [b], Andrea Vergani [b], Ahmed Abdelsalam [a,c], Emma Assi [a], Anna Maestroni [a], Vera Usuelli [a,b], Roberto Bassi [b], Ida Pastore [d], Jun Yang [e], Basset El Essawy [f,g], Khalid M. Elased [h], Gian Paolo Fadini [i], Elio Ippolito [a], Andy Joe Seelam [a], Marcus Pezzolesi [j], Domenico Corradi [k], Gian Vincenzo Zuccotti [l], Maurizio Gallieni [m,n], Marcello Allegretti [o], Monika Anna Niewczas [p], Paolo Fiorina [a,b,d,*]

[a] International Center for T1D, Centro di Ricerca Pediatrica Romeo ed Enrica Invernizzi, Dipartimento di Scienze Biomediche e Cliniche "L. Sacco", Università di Milano, Milan, Italy
[b] Nephrology Division, Boston Children's Hospital, Harvard Medical School, Boston, MA, USA
[c] Department of Biochemistry and Biotechnology, Heliopolis University, Cairo, Egypt
[d] Endocrinology Division, ASST Fatebenefratelli Sacco, Milan, Italy
[e] Institute of Organ Transplantation, Tongji Hospital and Medical College, Huazhong University of Science and Technology, Wuhan, China
[f] Medicine, Al-Azhar University, Cairo, Egypt
[g] Transplantation Research Center, Nephrology Division, Brigham and Women's Hospital, Boston, MA, USA
[h] Department of Pharmacology & Toxicology Boonshoft School of Medicine, Wright State University, Dayton, OH, USA
[i] Department of Medicine, University of Padua, Padua, Italy
[j] Division of Nephrology & Hypertension and Diabetes & Metabolism Research Center, University of Utah, Salt Lake City, UT, USA
[k] Pathology and Laboratory Medicine, University of Parma, Parma, Italy
[l] Centro di Ricerca Pediatrica Romeo ed Enrica Invernizzi, Dipartimento di Scienze Biomediche e Cliniche "L. Sacco", Università di Milano and Dipartimento di Pediatria, Ospedale dei Bambini Buzzi, Milan, Italy
[m] Dipartimento di Scienze Biomediche e Cliniche "L. Sacco", Università di Milano, Milan, Italy
[n] Nephrology and Dialysis Unit, ASST Fatebenefratelli-Sacco, Milan, Italy
[o] Dompé Farmaceutici SpA, L'Aquila, Italy
[p] Section on Genetics and Epidemiology, Joslin Diabetes Center, Boston, MA, USA

ARTICLE INFO

Article history:
Received 15 February 2021
Accepted 1 June 2021

Keywords:
CXCR1
CXCR2
CXCR1/2 blockade
Diabetic kidney disease
IL-8
Podocyte
Type 2 diabetes

ABSTRACT

Aims/hypothesis: Inflammation has a major role in diabetic kidney disease. We thus investigated the role of the IL-8-CXCR1/2 axis in favoring kidney damage in diabetes.
Methods: Urinary IL-8 levels were measured in 1247 patients of the Joslin Kidney Study in type 2 diabetes (T2D). The expression of IL-8 and of its membrane receptors CXCR1/CXCR2 was quantified in kidney tissues in patients with T2D and in controls. The effect of CXCR1/2 blockade on diabetic kidney disease was evaluated in db/db mice.
Results: IL-8 urinary levels were increased in patients with T2D and diabetic kidney disease, with the highest urinary IL-8 levels found in the patients with the largest decline in glomerular filtration rate, with an increased albumin/creatine ratio and the worst renal outcome. Moreover, glomerular IL-8 renal expression was increased in patients with T2D, as compared to controls. High glucose elicits abundant IL-8 secretion in cultured human immortalized podocytes *in vitro*. Finally, in diabetic db/db mice and in podocytes *in vitro*, CXCR1/2 blockade mitigated albuminuria, reduced mesangial expansion, decreased podocyte apoptosis and reduced DNA damage.
Conclusions/interpretation: The IL-8- CXCR1/2 axis may have a role in diabetic kidney disease by inducing podocyte damage. Indeed, targeting the IL-8-CXCR1/2 axis may reduce the burden of diabetic kidney disease.

1. Introduction

Diabetic kidney disease (DKD) is the primary cause of renal failure in the western world and is associated with an increased mortality rate in patients with type 2 diabetes [1], while the biological drivers of diabetic kidney disease are still far from being fully characterized [2,3]. We have recently underlined the role that inflammation plays in kidney damage

Abbreviations: ACR, albumin-to-creatinine ratio; DKD, Diabetic kidney disease; ESRD, End-stage renal disease.

* Corresponding author at: Nephrology Division, Boston Children's Hospital, Harvard Medical School, 300 Longwood Ave. Enders Building, Boston, MA 02115, USA.
E-mail address: paolo.fiorina@childrens.harvard.edu (P. Fiorina).

[1] These authors contributed equally to this work and should be considered co-first authors.

https://doi.org/10.1016/j.metabol.2021.154804
0026-0495/

during diabetic kidney disease [2,4–6]; previous reports have suggested a dysregulation of the pro-inflammatory chemokine IL-8 in type 1 and type 2 diabetes [7–9]. More recently, IL-8 levels have been correlated with levels of some biomarkers of both glomerular and tubular dysfunction in patients with type 2 diabetes [10]. However, the mechanism behind IL-8 action, as well as its targeting, has never been fully addressed. IL-8 is released by both immune and non-immune cells [11,12], including podocytes [13]. IL-8 is associated with insulin resistance and metabolic diseases [14] and its serum levels are increased in patients with type 2 diabetes [15]. IL-8 acts as a chemotactic factor that, in humans, strongly binds two membrane receptors: CXCR1 and CXCR2 [16,17]. Both receptors are expressed by several cell types, including immune cells [18] and podocytes [13]. We hypothesize that hyperglycemia triggers podocyte IL-8 production in patients with type 2 diabetes which, in turn, autocrinally activates death signals in CXCR1/CXCR2-expressing podocytes, thus favoring the development of diabetic kidney disease. We further hypothesize that pharmacological blockade of the IL-8-CXCR1/2 axis may prevent IL-8-mediated podocyte damage and progression of diabetic kidney disease.

2. Materials and methods

For a full description of the methods, please refer to the online electronic supplementary materials (ESM).

2.1. Human studies

This study included a cohort of 1247 patients from the Joslin Kidney Study in type 2 diabetes [1,19,20], 30 patients with type 2 diabetes from the Ospedale San Carlo (Milan, Italy) and 25 healthy subjects. Urinary IL-8 levels were measured by the Luminex® xMAP® technology. Morpho-pathological and immunofluorescence studies were performed with standard methods using antibodies listed in ESM Table 1.

2.1.1. Pathology and immunohistochemistry

Morpho-pathological studies and protein expression analysis on both human and murine tissues were performed by periodic acid Schiff staining (PAS) and immunohistochemistry evaluation respectively and following standard procedures. Briefly, 3 μm-thick sections of each paraffin-embedded biopsy were mounted on poly-L-lysine coated slides, deparaffinized and processed for antigen retrieval by dipping sections in 0.01 M citrate buffer, pH 6 for 10 min in a microwave oven at 650 W and for endogenous peroxidase activity inhibition by dipping sections in 3% (v/v) hydrogen peroxide for 10 min. Sections were then incubated with primary antibody at 4 °C for 18–20 h and processed for avidin-biotin complex procedure. Developing of immunoreactions was performed by 0.03% (v/v) 3,3'diaminobenzidine tetrahydrochloride and sections were then counterstained with Harris' hematoxylin. Pathological evaluation and Semiquantitative assessment of IL-8, CXCR1 and CXCR2 expression on tissue sections was performed by an expert pathologist and based on a grade scale from 0 to 4.

2.1.2. Immunofluorescence analysis

Immunofluorescence samples obtained from kidney biopsies were observed using a confocal system (LSM 510 Meta scan head integrated with the Axiovert 200 M inverted microscope; Carl Zeiss, Jena, Germany) equipped with a 63x/1.47 N.A. oil objective. Images were acquired in multitrack mode, using consecutive and independent optical pathways.

2.1.3. Real time PCR

Homogenized tissues were lysed using TRIzol® (Thermo Fisher Scientific) and total RNA was extracted using Direct-zol RNA Miniprep (Zymo Research, Irvine, California, USA). The cDNA was synthesized from 1 μg of total RNA using SuperScript IV (Thermo Fisher Scientific) according to the manufacturer's instructions. Twenty nanograms of each cDNA sample were used as template for the amplification reaction that was performed using SYBR® Green Mastermix (Bio-Rad, Hercules, California, USA) on a QuantStudio S6 Real Time System (Thermo Fisher Scientific). PCR amplifications were performed in triplicates and the $2^{-\Delta\Delta CT}$ method was used to calculate the relative amount of IL-8 (Taqman assays ID: hs00174103_m1), CXCR1 (Taqman assays ID: hs174146_m1) and CXCR2 (Taqman assays ID: hs174304_m1) using GAPDH expression (Taqman assays ID: Hs02758991_g1) as endogenous gene reference.

2.2. Murine studies

D2.BKS(D)-Leprdb/J (db/db) mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA). All mice were male and were cared for and used following the guidelines for animal care and housing of Boston Children's Hospital and Harvard Medical School. Institutional Animal Care and Use Committee approved the protocol. Blockade of CXCR2 in db/db mice was performed *via* intraperitoneal injection of a CXCR1/2 blocker [21], (Reparixin, kindly provided by Dompé Farmaceutici SpA) at a dose of 15 mg/kg bid from week 7 to week 25 of age as per manufacturer's instructions. The specific activity of Reparixin (formerly repertaxin), the CXCR1/2 non allosteric antagonist used in our study, has been already tested and demonstrated in other studies [21,22]. Twenty db/db mice were included in the reparixin-treated group, while ten db/db mice were used as controls. All control mice were treated twice per day by i.p. vehicle injections for 18 weeks, thus paralleling the schedule of interventions followed for mice of the CXCR1/2 antagonist-treated group. Fasting blood glucose was monitored twice per week starting when db/db mice were 7–8-week-old in order to confirm diabetes onset and permanence. At the designated time point, fasting glycemia was measured and mice were then anaesthetized to obtain blood samples to collect serum/plasma and kidneys for histology studies. Kidney function was assessed by measuring creatinine through the Adaltis PCHEM-1 technical instrument. KC urinary and serum levels were assessed by Luminex Bio-Plex Pro Mouse KC/CXCL1 (#12002246 Bio-Rad).

2.2.1. Murine kidney pathology

Kidney tissue was collected from db/db mice at 8, 15 and 25 weeks and fixed in 4% (w/v) buffered paraformaldehyde, dehydrated and paraffin embedded. Immunohistochemistry was performed with 5-μm-thick formalin-fixed, paraffin-embedded tissue sections as described above. AxioVision software 4.3 was used to record images (Carl Zeiss). PAS staining was employed to detect mesangial volume while Van Gieson staining was used to detect matrix deposition. Evaluation of mesangial matrix was performed electronically by a macro built on the AxioVision analysis module (Carl Zeiss SpA, Thornwood, NY). Briefly, glomeruli were identified as region of interest (ROI) and mesangium highlighted by a colour threshold procedure. Binary images were then generated and mesangium automatically calculated as percentage of the glomerular area. A semiquantitative morphometric analysis was performed by a blinded pathologist in at least 40 glomeruli per sample. Each glomerulus was given a score from 1 to 4 as following: 1, no mesangial matrix expansion; 2, increase in mesangial matrix and/or cellularity; 3, the expanded mesangial area exceeded the mean area of a capillary lumen with distorted/compressed capillaries; and 4, the mesangium is expanded and/or sclerosed and/or with capillary lumens completely collapsed. Podocyte apoptosis was evaluated in deparaffinized db/db kidney tissue sections by the Click-iT Plus TUNEL Assay (Thermo Fisher Scientific, Waltham, MA USA) using an Alexa Fluor® picolyl azide fluorescent dye and following manufacturer's protocol. Apoptosis was also assessed by using a polyclonal antibody against Caspase 3 (Novus Biologicals, Littleton, CO) for immunohistochemistry analysis on 4% buffered PFA-fixed, paraffin-embedded samples. At least 40 glomeruli per sample were analyzed and number of positive nuclei per glomerulus was assessed by using a score from 0 (absence) to 3 [5]. Images were acquired by confocal scanning laser microscopy (Leica TCS SP8; Leica Microsystems, Wetzlar, Germany) with a 40× objective.

2.3. In vitro studies

Immortalized podocytes cells were kindly provided by Dr. M. A. Saleem and cultured as previously described [23]. Podocytes were cultured and allowed to grow to 80% confluence at 33 °C. Cells were then thermoshifted to 37 °C and incubated for 14 days before starting experiments. Recombinant IL-8 protein (rIL-8, ChinaPeptides, Shanghai, China) challenge was performed by adding rIL-8100 ng/ml to the culturing medium. Podocyte CXCR1/2 blockade was achieved by incubating the cells in a medium containing Reparixin 100 nM. Glucose challenge was performed by culturing cells in high glucose (30 mM), low glucose (5 mM), mannitol (18.9 mM plus glucose 11.1 mM).

2.3.1. Transcriptome profiling

Total RNA was isolated from podocytes cultured with/without rIL-8 or with/without 35 mM glucose using the RNeasy Mini Kit (Qiagen, Valencia, CA) with on-column DNase I digestion. Next, 3 μg total RNA from each sample was reverse-transcribed using the RT2 First Strand kit (C-03; SABiosciences, Frederick, MD). We used the Human Nephrotoxicity RT2 Profiler PCR Arrays (PAHS-094ZA).

2.4. Statistical analysis

Prism v. 7.0 (GraphPad Software, La Jolla, CA, USA) was used as statistical software for data analysis. A D'Agostino & Pearson normality test was performed, and parametric *t*-test or non-parametric Mann-Whitney test were performed according to data distribution. Chi-square test with Yates' correction was performed for the analysis of categorical variables. Albumin/creatine ratios and glomerular filtration rate slopes were compared by a Kolmogorov-Smirnov test. All data are presented as mean ± SEM. All p values <0.05 were considered significant.

3. Results

3.1. IL-8 urinary levels are elevated in patients with type 2 diabetes and DKD

In order to understand if the IL-8-CXCR1/2 axis is overactive in DKD, we first quantified IL-8 in the urine of patients included in the Joslin Kidney Study that were affected by type 2 diabetes at different stages of DKD. IL-8 levels resulted increased in patients with type 2 diabetes as compared to healthy subjects (Fig. 1A). Of note, IL-8 was higher in the urine of microalbuminuric or proteinuric as compared to normoalbuminuric patients (Fig. 1B) without any correlation observed with IL-8 serum levels (ESM Fig. 1A–B). Importantly, in a sub-group of 368 randomly selected patients with type 2 diabetes and DKD that were followed for a period of 5 years (ESM Table 2), those with detectable urinary IL-8 manifested a worse renal outcome, with significantly increased albumin-to-creatinine ratio (ACR) (Fig. 1C) and faster decline in glomerular filtration rate (GFR) over time (Fig. 1D). Furthermore, 41 patients (~20%) that had a IL-8 positive urine showed a composite renal outcome as defined by the Food and Drug Administration (ESRD – End Stage Renal Disease – death for any cause, or 30% of kidney function loss), while only 14 (9%) of patients with no urinary IL-8 experienced a composite renal outcome (Fig. 1E and ESM Table 3). Among patients with type 2 diabetes, those microalbuminuric with a detectable urinary IL-8 showed higher ACR values and a higher GFR slope as compared to urinary IL-8 negative patients (Fig. 1F and G), while 36 (20%) and 14 (11%) of microalbuminuric patients with positive and negative urines for IL-8, respectively, suffered of a composite renal outcome (Fig. 1H and ESM Table 3). Finally, normoalbuminuric patients whose urine was positive for IL-8 had increased ACR (Fig. 1I) and unchanged GFR (Fig. 1J) as compared to urinary IL-8 negative patients. Remarkably, no urinary IL-8 negative normoalbuminuric patients suffered from a composite renal outcome, as compared to 5 (13%) subjects that resulted positive for urinary IL-8 (Fig. 1K and ESM Table 3). Patients enrolled in this study were on antidiabetic medications and RAAS inhibitors (ESM Table 4), with no particular association between IL-8 levels and medications received (*e.g.*, RAAS inhibitors $p = 0.5$, anti-diabetic oral agents $p = 0.5$). No significant associations were found between urinary IL-8 and worse renal outcome, increased ACR and faster decline in GFR after adjusting for demographic factors and clinical parameters (age, gender, ethnicity, duration of diabetes and ongoing treatments) (ESM Table 5). Overall, these findings suggest a potential link between IL-8 urinary levels and DKD in patients with type 2 diabetes.

3.2. IL-8, CXCR1 and CXCR2 glomerular expression is dysregulated in patients with type 2 diabetes and DKD

We then assessed IL-8, CXCR1 and CXCR2 expression on 30 renal biopsies from patients with type 2 diabetes at different stages of DKD and on healthy renal tissue obtained from 10 renal cancer nephrectomies used as controls. Quantitative RT-PCR revealed enhanced expression of IL-8, but not of CXCR1 or CXCR2, in kidney samples of patients with type 2 diabetes and DKD as compared to the controls (Fig. 2A–C). Among the samples, a positivity for IL-8 and CXCR1, but not of CXCR2, protein immunostaining was evident in biopsies of patients with type 2 diabetes and DKD, as compared to controls (Fig. 2D–F). More interestingly, when kidney samples obtained from T2D patients were stratified according to their DKD stage and compared to the control samples, we observed a marked increase of IL-8 protein expression in biopsies with mesangial expansion, while a progressive decrease was evident with the advancing of the DKD stage, following the loss of cellularity of the kidney parenchyma, the onset of fibrosis and the late-stage scarring (Fig. 2G1–G4, H). CXCR1 displayed a similar pattern, with protein being more expressed in the glomeruli affected by mesangial expansion than in those of controls, while CXCR2 expression did not show any difference (Fig. 1I1–I4, J, K1–K4, L). The increase of both IL-8 and CXCR1 was not paralleled by an increase in glomerular macrophage infiltration (Fig. 2: M1-M4, N). A dysregulation of IL-8 and CXCR1 expression seems to be evident at glomerular level in patients with type 2 diabetes at the early stages of DKD.

3.3. Blockade of the IL-8-CXCR1/2 axis reduces progression of DKD in diabetic db/db mice

To establish the blueprint for the relevance of targeting the IL-8-CXCR1/2 axis, we first investigated the status of the aforementioned axis in db/db mice, which develop experimental DKD. Db/db mice lack the expression of a CXCR1 homologue receptor and KC is the murine homologue of IL-8 [24]. First, we tested the expression of both KC and CXCR2 in kidney of db/db mice and found that both proteins, mainly expressed by podocytes (ESM Fig. 2A), are detectable in the kidney of db/db mice at 8 weeks of age and that their expression increased with the development of DKD (Fig. 3A1–A3, B and D1–D3, E), including that of circulating KC (Fig. 3C). We then investigated the effect of an IL-8-CXCR1/2 axis blockade on renal function *in-vivo* by administering a non-competitive blocker of human and murine IL-8 receptors that allosterically binds to CXCR1/CXCR2 (*e.g.* Reparixin), thus preventing their activation by IL-8/KC. After 18 weeks of treatment, CXCR2 blockade remarkably reduced urinary albumin excretion in treated mice as compared to the untreated group, used as controls (Fig. 3F). A decrease in the mesangial volume/expansion was also evident in the glomeruli of db/db mice treated with the CXCR2 blocker after 18 weeks (Fig. 3G1–G2 and H) and it was associated although to a lesser degree with a reduction in matrix deposition (Fig. 3I1–I2 and 3J). Interestingly, no differences were observed in glycemic levels, body weight and renal function between treated mice and controls (ESM Fig. 2B and C–D), although body weight showed a slight reduction after 9 weeks of treatment with CXCR2 blocker, indicating that the observed improvement in renal function is not mediated by glycometabolic or morphological alterations. Indeed, blood glucose levels, urinary albumin excretion and

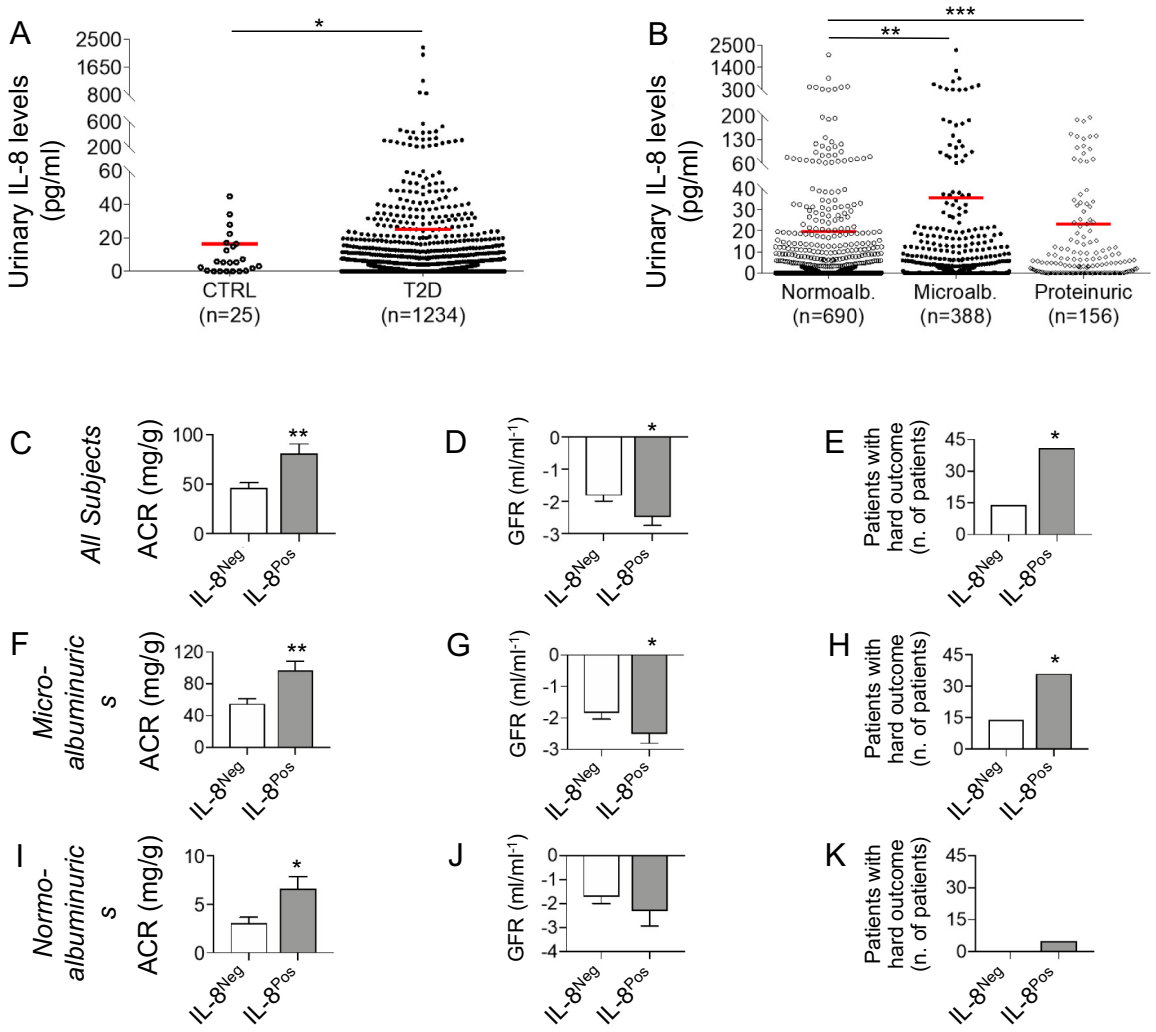

Fig. 1. Urinary IL-8 levels are increased in patients with type 2 diabetes and DKD.
Urinary IL-8 levels were increased in patients with T2D as compared to healthy controls (A). Among T2D patients, those with microalbuminuria or proteinuria showed higher urinary IL-8 levels than normoalbuminuric patients (B). IL-8 presence in urine ($IL\text{-}8^{Pos}$) was associated to impaired renal function – as expressed by increased urinary albumin-to-creatinine ratio and by a more negative glomerular filtration rate slope – and to worse outcomes in patients with type 2 diabetes and DKD (C–E). Data were confirmed in patients with type 2 diabetes and micro- (F–H) or normo-albuminuria (I–K).

body weight were significantly increased in db/db mice with DKD at 25 weeks of age as compared to those of 8 weeks of age without signs of DKD (ESM Fig. 2E–H). We then assessed the effect of the CXCR1/2 blockade on *in-vivo* podocyte viability by a TUNEL assay and Caspase 3 immunostaining performed on renal tissues of diabetic db/db mice. A decreased apoptosis was observed in the glomeruli obtained from treated db/db mice as compared to the controls (Fig. 3K1–K2, L and M1–M2, N). These results suggest a protecting role of the CXCR2 blockade on renal morphology/function and podocyte viability in db/db diabetic mice.

3.4. The IL-8-CXCR1/2 axis is activated by high glucose in human podocyte cells in vitro

We then aimed at characterizing the IL-8-CXCR1/2 axis activity in human podocytes by using a conditionally immortalized human podocyte cell line *in vitro*. Both CXCR1 and CXCR2 expressions were clearly noticeable in cultured podocytes following immunofluorescence staining (Fig. 4A). At flow cytometric analysis, both receptors showed a weak but consistent expression in immortalized podocytes (ESM Fig. 3A). To investigate the role of the IL-8-CXCR1/2 axis in diabetic-like conditions, podocytes were cultured under high glucose concentrations (30 mM) and the podocytes CXCR1/2 expression or podocytes IL-8 release into culturing medium were measured. Normal glucose (10 mM) or equiosmolar mannitol (30 mM) concentrations were used as controls during 72 h of culture. We observed a remarkable increase in IL-8 release from podocytes cultured in high-glucose as compared to those grown in normal glucose or with mannitol (Fig. 4B). A persistent though unchanged expression of CXCR1 and CXCR2 was observed on cultured cells by either flow cytometric evaluation or western blot analysis (ESM Fig. 3B–H). Finally, we mechanistically addressed the effect of the IL-8-CXCR1/2 axis activity on podocyte DNA stability. To this aim, we exposed podocyte cells *in-vitro* to a recombinant IL-8 protein (rIL-8) alone or in combination with a CXCR1/2 blocker and evaluated the induced cell DNA injury as revealed by increased levels of the phosphorylated histone H2A.X variant (γ-H2A.X), used as a DNA strand marker break occurrence. An increase in the γ-H2A.X/H3 histone ratio was found when podocytes were challenged with rIL-8 as compared to untreated cells through western blot analysis and the addition of a CXCR1/2 blocker to the culture restored the γ-H2A.X/H3 histone ratio that was observed in untreated cells (Fig. 4C and D). This effect was also visible at the immunofluorescence analysis, where the generation

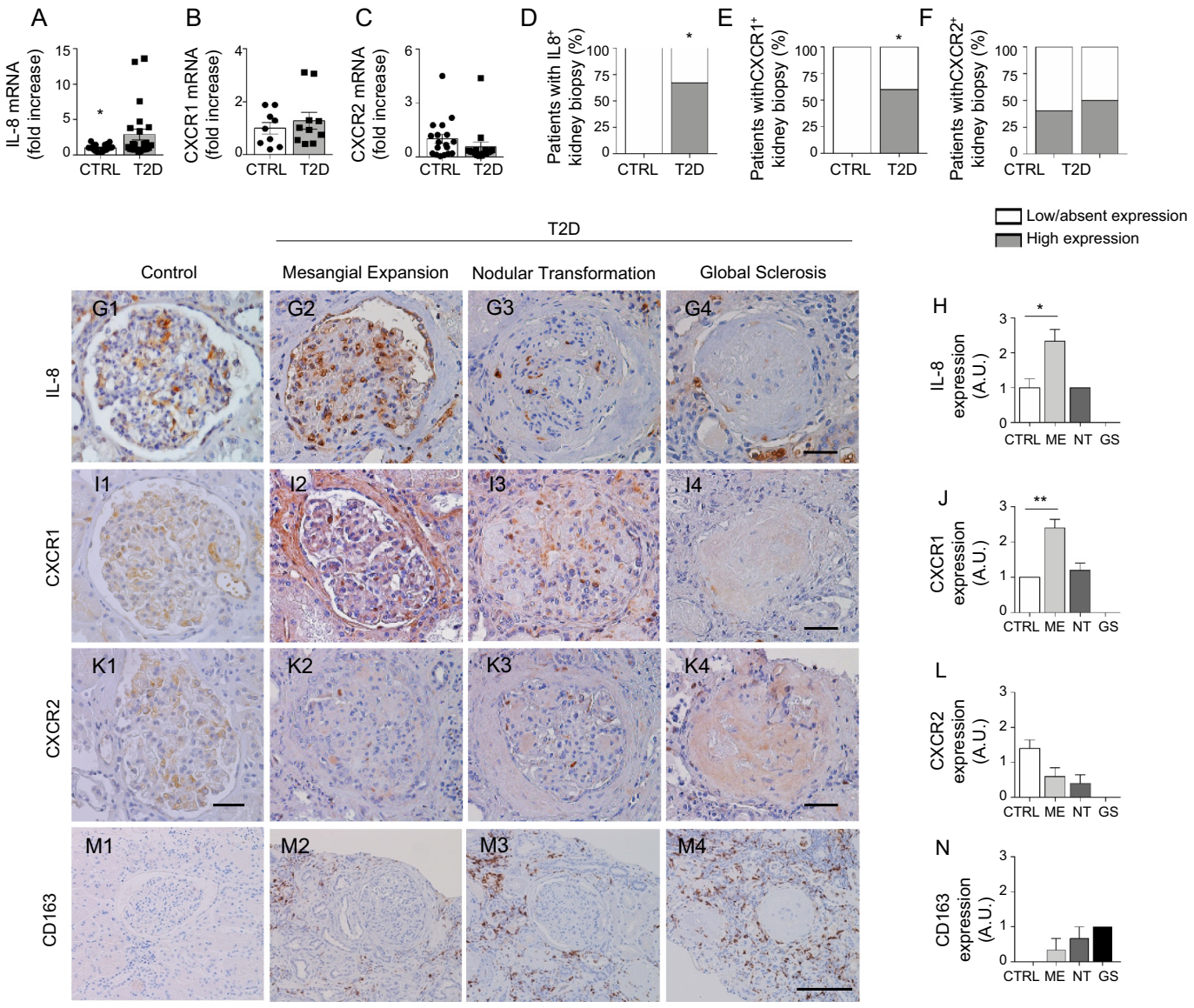

Fig. 2. IL-8-CXCR1/2 axis is dysregulated in patients with type 2 diabetes and DKD.
IL-8 mRNA, but not that of CXCR1 or CXCR2, was more expressed in the renal samples of patients with type 2 diabetes than in controls, while no differences in IL-8, CXCR1 or CXCR2 expression were observed at the immunohistochemical staining (A–C). A higher percentage of patients with type 2 diabetes showed high expression of IL-8, CXCR1 and CXCR2, the latter to a lesser extent, in kidney biopsies as compared to controls (D–F). When comparing different stages of DKD at protein level, both IL-8 and its receptors resulted more abundant in glomerular tissues of patients with type 2 diabetes and mesangial expansion as compared to controls (G–L). More advanced stages of DKD were associated to reduced levels of IL-8, CXCR1 and CXCR2 protein expression, in conjunction with the loss of cellularity of the kidney parenchyma and the onset of fibrosis (scale bar, 25 μm) (G1–G4, H, I1–I4, J, K1–K4, L). No difference was observed with regard of macrophage infiltration (CD163 staining) in glomerular tissues of patients with type 2 diabetes and mesangial expansion as compared to controls (M1–M4, N). Data are expressed as mean ± SEM. ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).
Abbreviations. T2D: type 2 diabetes; CTRL: healthy controls; ACR: albumin-to-creatinine ratio; GFR: glomerular filtration rate slope; IL-8^{Pos}: IL-8 positive urine; IL-8^{Neg}: IL-8 negative urine; ME: mesangial expansion; NT: nodular transformation; GS: global sclerosis; A.U.: arbitrary unit.

of DNA damage-associated γ-H2A.X-containing nuclear structures (γ-H2A.X *foci*) were found to be increased in the nuclei of rIL-8-cultured podocyte cells as compared to untreated cells and to the cells treated with rIL-8 in combination with the CXCR1/2 blocker (ESM Fig. 4A–C). A transcriptome profile of the rIL-8-cultured podocyte cells revealed a two-fold upregulation of transcripts related to inflammatory/oxidative stress injury (*CCL3*, *CXCL10*, *NOX4*) and podocytes dysfunction/apoptosis (*NPHS2*, *EGF*, *BMP4*, *KLK1*), while a downregulation was evident for transcripts with nephroprotective effects (*GSTK1*, *SOD2* and *TIMP1*), (Fig. 4E). This pattern was only in part paralleled in high glucose-cultured podocytes, with gene expression of BMP4 being upregulated and that of SOD2 being downregulated (Fig. 4E). Despite this, a unique transcriptome profile was evident in rIL-8-cultured podocytes, which confirms an IL-8 independent effect on podocytes and may represent an IL-8 specific signature (Fig. 4E). Overall, these findings suggest a glucose-dependent IL-8-CXCR1/2 axis activation in podocytes that may autocrinally exert a detrimental and toxic effect on podocytes (Fig. 4F).

4. Discussion

Inflammation is a recognized key-player in driving the onset of and in sustaining DKD [2,25,26]. IL-8 is a pro-inflammatory chemokine that is increased in the serum of patients with type 2 diabetes [15,27]. Here, we show that IL-8 is also increased in urine of patients with type 2 diabetes and DKD that levels of urinary IL-8 are strongly associated

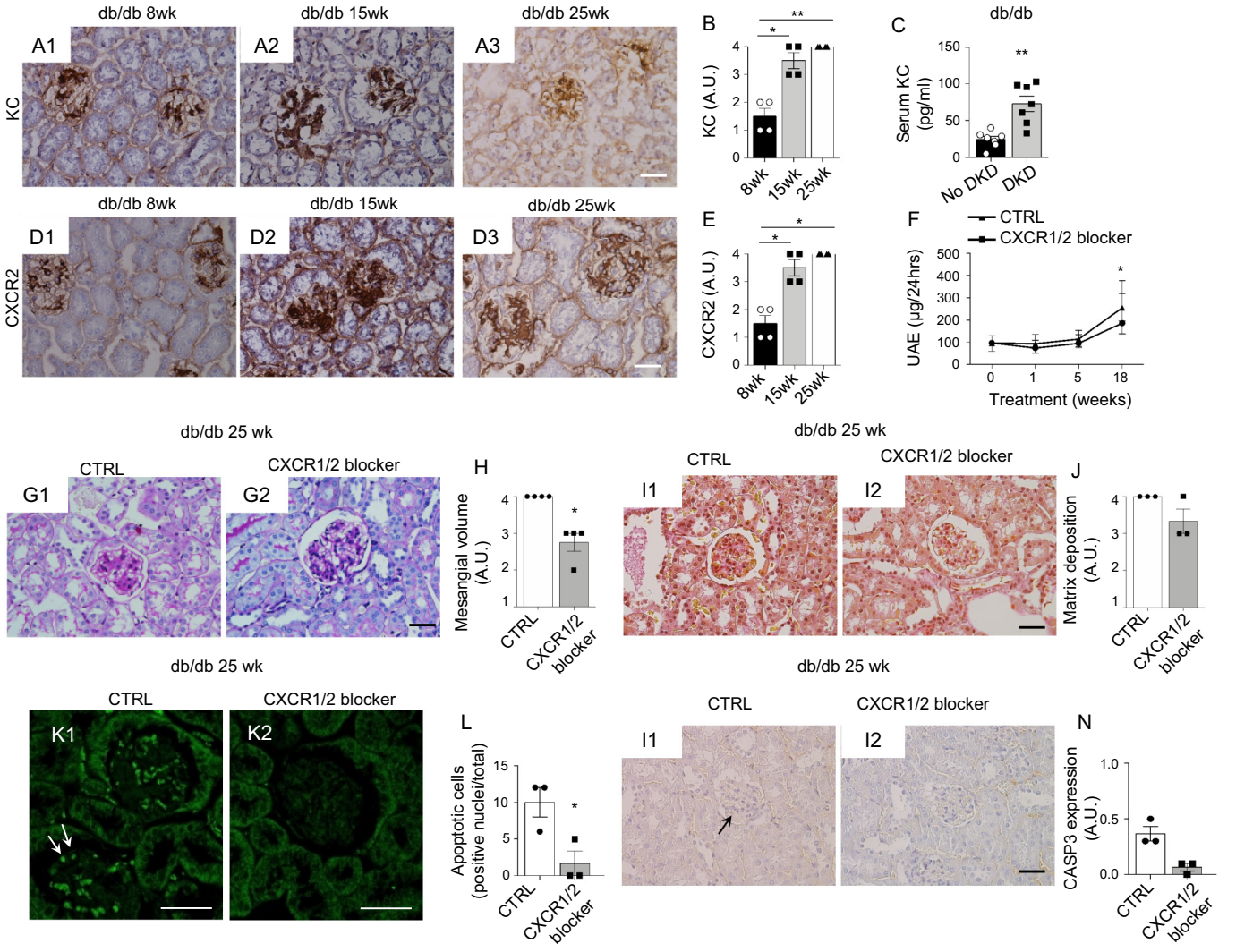

Fig. 3. Blockade of the IL-8-CXCR1/2 axis reduces progression of DKD in db/db diabetic mice. KC – the murine homologue of IL-8 –is expressed in the glomeruli of diabetic db/db mice and its expression increases with DKD progression (scale bar, 50 µm) (A1–A3, B). KC serum levels are detectable in db/db mice and increase in those with DKD (18 weeks-old) (C). KC-receptor CXCR2 is expressed in the glomeruli of diabetic db/db mice and its expression increases with DKD progression (scale bar, 50 µm) (D1–D3, E). The blockade of murine CXCR2 by a CXCR2 blocker treatment reduced the urinary albumin excretion as compared to untreated mice used as controls ($n = 10$, CTRL), (F). Periodic acid Schiff (PAS) and Van Gieson staining showed decreased mesangial volume and matrix deposition in diabetic db/db mice that were administered a CXCR2 blocking agent as compared to untreated mice (CTRL, scale bar, 50 µm) (G1–G2, H and I1–I2, J). CXCR2 blockade resulted in reduced apoptosis detected by using TUNEL (positive nuclei/total nuclei) and Caspase 3 immunostaining in CXCR2-blocker treated mice at the glomerular level ($n = 3$) (scale bar, 50 µm) (K1–K2, L and M1–M2, N). Data are expressed as mean ± SEM. Urinary albumin is expressed as median ± interquartile range ($^{*}p < 0.05$, $^{**}p < 0.01$).
Abbreviations. UAE: urinary albumin excretion; CTRL: untreated mice; A.U.: arbitrary unit.

with a reduced renal function, a faster GFR decline and worse clinical outcomes. The small proportion of subjects analyzed within the "Joslin Kidney Study" revealed an interesting role for IL-8 in DKD. This has been recently confirmed in a small pilot study in which IL-8 urinary and circulating levels were found to be associated with an increase in markers of podocyte injury and tubular dysfunction in patients with DKD [10]. However, these findings are preliminary and need to be confirmed in larger studies. We also detected the expression of IL-8 and of its receptors in human and murine glomeruli, while immortalized human podocyte cells showed CXCR1 and CXCR2 expression. Interestingly, IL-8-release is strongly enhanced during high-glucose challenge *in vitro*. These findings suggest a possible autocrine detrimental action of the IL-8-CXCR1/2 axis on podocytes during hyperglycemia, which in turn may boost the activation of the axis itself and, in addition to other toxic and pro-inflammatory factors, may promote kidney damage. Overall, the altered IL-8 urinary levels detected in patients with type 2 diabetes, the expression of IL-8 receptors CXCR1/2 in renal tissue and the increased IL-8 release observed in high glucose-cultured podocytes confirm a dysregulation of the IL-8-CXCR1/2 axis in DKD. However, we acknowledge that activation of the IL-8-CXCR1/2 axis is not the sole factor involved in podocyte damage/loss in DKD, but a combination of several factors, including high glucose toxicity, IL-8 and additional serum factors may play a significant role. To test this hypothesis, we assessed the effect of a CXCR1/2 blocker treatment on renal function in diabetic db/db mice that spontaneously develop DKD. The blockade of the IL-8-CXCR1/2 axis improved kidney function in diabetic db/db mice and reduced mesangial expansion, with no changes in glycemia levels, implying the occurrence of both a morphological and a functional effect. The beneficial effects of inhibiting the IL-8-CXCR1/2 axis has been also confirmed in db/db mouse with diabetes treated with a CXCL8 antagonist [28] and in another murine model of DKD, in which mice injected with streptozotocin and fed a high-fat diet treated with the aforementioned CXCL8 antagonist, demonstrated protection from macrophage renal infiltration, podocyte loss and glomerulosclerosis [29]. Indeed, a protective effect on kidney function in a DSS-induced renal injury model has been also observed in the CXCR2$^{-/-}$ mouse, in which the IL-8-CXCR1/2 signaling is abrogated and circulating levels of IL-8 were decreased [30]. Given that CXCR2 and – to a lesser extent – CXCR1

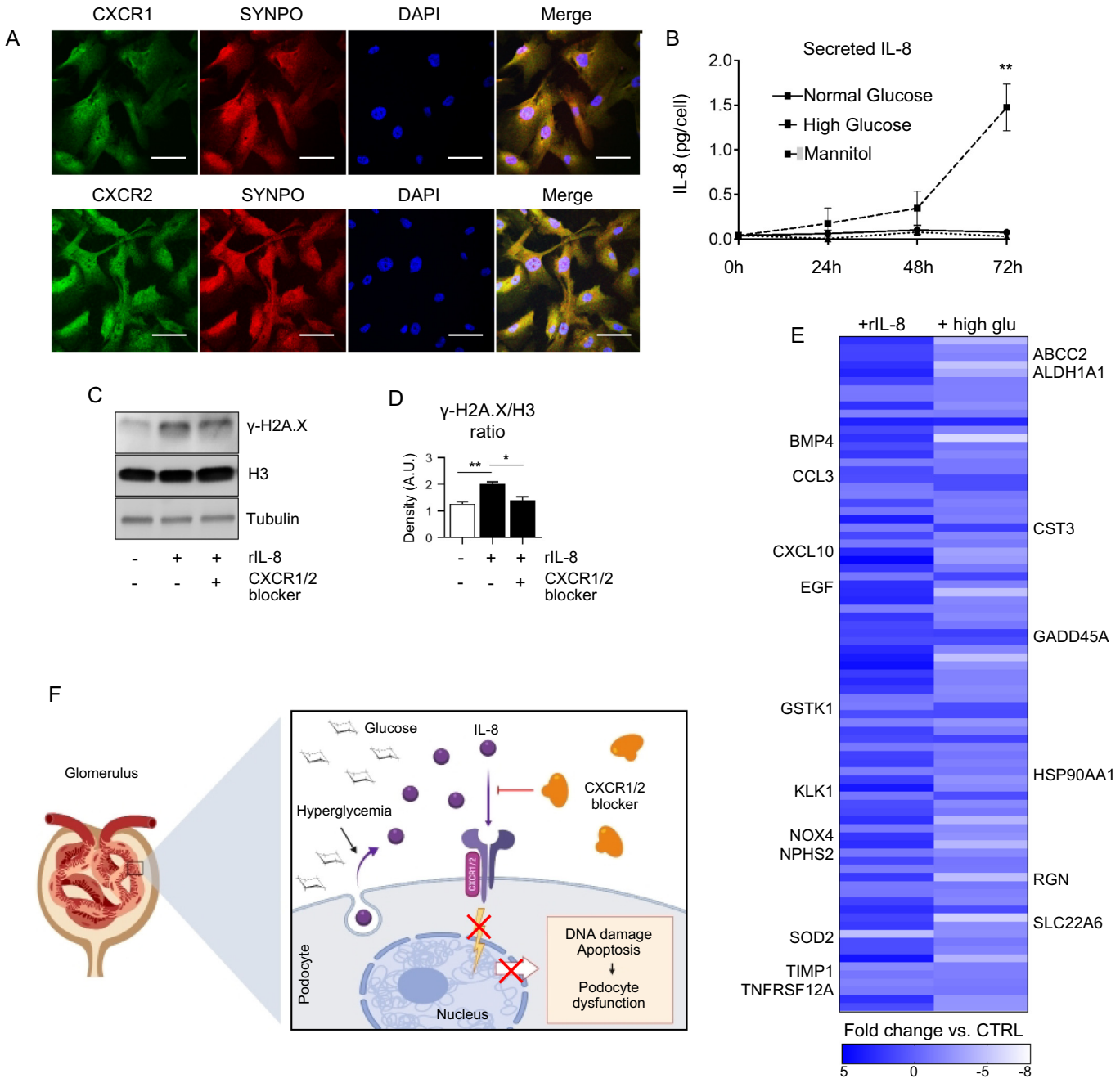

Fig. 4. Blockade of the IL-8-CXCR1/2 axis reduces IL-8 mediated DNA injury in immortalized podocyte cells *in vitro*.
A human immortalized podocyte cell line cultured *in vitro* expressed both IL-8 receptors (green) (scale bar, 70 μm) (A). Culturing such cells in high glucose induced a sharp increase in IL-8 podocyte release into the medium (n = 3) (B). Blockade of the IL-8-CXCR1/2 axis also reduced the observed podocyte DNA injury occurring when recombinant IL-8 was added to the podocyte culture, as shown by γ-H2A.X/H3 histone ratio at western blot analysis (n = 3) (C and D). Transcriptome analysis revealed a distinctive signature in IL-8 cultured human podocytes, in part shared with that observed during high glucose exposure (n = 3) (E). We propose a model whereby hyperglycemia induces the release of IL-8 from podocytes, which in turn triggers a CXCR1/2-mediated signaling in podocytes, thus originating an autocrine detrimental loop that generates podocyte DNA damage, cell death and renal function impairment (F). Data are expressed as mean ± SEM. (*$p < 0.05$, **$p < 0.01$)
Abbreviations. SYNPO, synaptopodin.

may also act as receptors for CXCL1, CXCL2, CXCL3, CXCL4, CXCL5, CXCL6 and CXCL7 to promote migration/recruitment of immunocytes (macrophages, neutrophils) to inflammatory sites, CXCR1/2-blockade may exert some positive effects by halting the CXCLs-CXCR1/2 signaling in inflammation [31]. In particular, the CXCL7/CXCR2 axis may offer an off-target effect of CXCR1/2 blockers in diabetic kidney disease [32]. Finally, when culturing *in vitro* podocytes with IL-8, DNA injuries occurred, and these injuries were prevented by CXCR1/2 blockade. Interestingly, high glucose exposure as well as IL-8 challenge partially shared a podotoxic signature in human podocytes *in vitro*, with IL-8 showing an independent effect on several nephrotoxic pathways, thus supporting a link between dysregulated IL-8 signaling and podocytes loss in the onset of DKD. Mechanistically, we propose that hyperglycemia stimulates IL-8 podocyte release and that the increased levels of IL-8 may trigger CXCR1/2 signaling, thus generating an autocrine detrimental loop that induces podocyte DNA damage, cell death and renal function impairment (Fig. 4F). In conclusions, our results suggest a role for the IL8-CXCR1/2 axis in DKD onset and that IL-8-CXCR1/2 axis

may represent a novel, therapeutic target to treat type 2 diabetes patients and prevent/treat DKD.

Funding/financial support

P.F. is supported by an Italian Ministry of Health grant RF-2016-02362512 and by Linea-2 2019 funding from Università di Milano. F.D. is the recipient of the EFSD/JDRF/Lilly Programme on Type 1 Diabetes Research 2019.

Data availability

The data generated during and/or analyzed during the current study is available from the corresponding author on reasonable request.

Ethics declarations

M.A. is employee of Dompé Farmaceutici SpA, Italy. All other authors declare no present or potential conflicts of interest. This study was performed without the support of any external funding source or study sponsor.

CRediT authorship contribution statement

C.L. F.R. and F.D. designed the study, performed experiments, analyzed data, and wrote the paper; M.BN., A.A., I.P., E.A., A.M., E.I., AJ.S. and D.C. performed experiments and analyzed data; E.C.L., S.D., A.V., V.U., GP.F. and KM.E. designed and performed animal studies; J.Y., B. EE., M.P., GV.Z., M.G., M.A. and MA.N. coordinated the research and edited the paper; P.F. conceived the idea, designed the study and wrote and edited the paper.

Acknowledgments

We thank Fondazione Romeo and Enrica Invernizzi for the extraordinary support.

References

[1] Nowak N, Skupien J, Smiles AM, Yamanouchi M, Niewczas MA, Galecki AT, et al. Markers of early progressive renal decline in type 2 diabetes suggest different implications for etiological studies and prognostic tests development. Kidney Int. 2018;93:1198–206.
[2] Niewczas MA, Pavkov ME, Skupien J, Smiles A, Md Dom ZI, Wilson JM, et al. A signature of circulating inflammatory proteins and development of end-stage renal disease in diabetes. Nat Med. 2019;25:805–13.
[3] Colhoun HM, Marcovecchio ML. Biomarkers of diabetic kidney disease. Diabetologia. 2018;61:996–1011.
[4] Fiorina P, Ansari MJ, Jurewicz M, Barry M, Ricchiuti V, Smith RN, et al. Role of CXC chemokine receptor 3 pathway in renal ischemic injury. J Am Soc Nephrol. 2006;17:716–23.
[5] Fiorina P, Vergani A, Bassi R, Niewczas MA, Altintas MM, Pezzolesi MG, et al. Role of podocyte B7-1 in diabetic nephropathy. J Am Soc Nephrol. 2014;25:1415–29.
[6] D'Addio F, Trevisani A, Ben Nasr M, Bassi R, El Essawy B, Abdi R, et al. Harnessing the immunological properties of stem cells as a therapeutic option for diabetic nephropathy. Acta Diabetol. 2014;51:897–904.
[7] Wolkow PP, Niewczas MA, Perkins B, Ficociello LH, Lipinski B, Warram JH, et al. Association of urinary inflammatory markers and renal decline in microalbuminuric type 1 diabetics. J Am Soc Nephrol. 2008;19:789–97.
[8] Tashiro K, Koyanagi I, Saitoh A, Shimizu A, Shike T, Ishiguro C, et al. Urinary levels of monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8), and renal injuries in patients with type 2 diabetic nephropathy. J Clin Lab Anal. 2002;16:1–4.
[9] Singh K, Agrawal NK, Gupta SK, Sinha P, Singh K. Increased expression of TLR9 associated with pro-inflammatory S100A8 and IL-8 in diabetic wounds could lead to unresolved inflammation in type 2 diabetes mellitus (T2DM) cases with impaired wound healing. J Diabetes Complications. 2016;30:99–108.
[10] Milas O, Gadalean F, Vlad A, Dumitrascu V, Velciov S, Gluhovschi C, et al. Pro-inflammatory cytokines are associated with podocyte damage and proximal tubular dysfunction in the early stage of diabetic kidney disease in type 2 diabetes mellitus patients. J Diabetes Complications. 2020;34:107479.
[11] Baggiolini M, Clark-Lewis I. Interleukin-8, a chemotactic and inflammatory cytokine. FEBS Lett. 1992;307:97–101.
[12] Moore BB, Kunkel SL. Attracting attention: discovery of IL-8/CXCL8 and the birth of the chemokine field. J Immunol. 2019;202:3–4.
[13] Huber TB, Reinhardt HC, Exner M, Burger JA, Kerjaschki D, Saleem MA, et al. Expression of functional CCR and CXCR chemokine receptors in podocytes. J Immunol. 2002;168:6244–52.
[14] Barchetta I, Cimini FA, Capoccia D, De Gioannis R, Porzia A, Mainiero F, et al. WISP1 is a marker of systemic and adipose tissue inflammation in dysmetabolic subjects with or without type 2 diabetes. J Endocr Soc. 2017;1:660–70.
[15] Cimini FA, Barchetta I, Porzia A, Mainiero F, Costantino C, Bertoccini L, et al. Circulating IL-8 levels are increased in patients with type 2 diabetes and associated with worse inflammatory and cardiometabolic profile. Acta Diabetol. 2017;54:961–7.
[16] Nasser MW, Raghuwanshi SK, Grant DJ, Jala VR, Rajarathnam K, Richardson RM. Differential activation and regulation of CXCR1 and CXCR2 by CXCL8 monomer and dimer. J Immunol. 2009;183:3425–32.
[17] Petering H, Gotze O, Kimmig D, Smolarski R, Kapp A, Elsner J. The biologic role of interleukin-8: functional analysis and expression of CXCR1 and CXCR2 on human eosinophils. Blood. 1999;93:694–702.
[18] Russo RC, Garcia CC, Teixeira MM, Amaral FA. The CXCL8/IL-8 chemokine family and its receptors in inflammatory diseases. Expert Rev Clin Immunol. 2014;10:593–619.
[19] Ihara K, Skupien J, Kobayashi H, Md Dom ZI, Wilson JM, O'Neil K, et al. Profibrotic circulating proteins and risk of early progressive renal decline in patients with type 2 diabetes with and without albuminuria. Diabetes Care. 2020;43:2760–7.
[20] Pezzolesi MG, Poznik GD, Skupien J, Smiles AM, Mychaleckyj JC, Rich SS, et al. An intergenic region on chromosome 13q33.3 is associated with the susceptibility to kidney disease in type 1 and 2 diabetes. Kidney Int. 2011;80:105–11.
[21] Bertini R, Allegretti M, Bizzarri C, Moriconi A, Locati M, Zampella G, et al. Noncompetitive allosteric inhibitors of the inflammatory chemokine receptors CXCR1 and CXCR2: prevention of reperfusion injury. Proc Natl Acad Sci U S A. 2004;101:11791–6.
[22] Casilli F, Bianchini A, Gloaguen I, Biordi L, Alesse E, Festuccia C, et al. Inhibition of interleukin-8 (CXCL8/IL-8) responses by repertaxin, a new inhibitor of the chemokine receptors CXCR1 and CXCR2. Biochem Pharmacol. 2005;69:385–94.
[23] Saleem MA, O'Hare MJ, Reiser J, Coward RJ, Inward CD, Farren T, et al. A conditionally immortalized human podocyte cell line demonstrating nephrin and podocin expression. J Am Soc Nephrol. 2002;13:630–8.
[24] Cacalano G, Lee J, Kikly K, Ryan AM, Pitts-Meek S, Hultgren B, et al. Neutrophil and B cell expansion in mice that lack the murine IL-8 receptor homolog. Science. 1994;265:682–4.
[25] Hickey FB, Martin F. Role of the immune system in diabetic kidney disease. Curr Diab Rep. 2018;18:20.
[26] Tang SCW, Yiu WH. Innate immunity in diabetic kidney disease. Nat Rev Nephrol. 2020;16:206–22.
[27] Giannattasio S, Corinaldesi C, Colletti M, Di Luigi L, Antinozzi C, Filardi T, et al. The phosphodiesterase 5 inhibitor sildenafil decreases the proinflammatory chemokine IL-8 in diabetic cardiomyopathy: in vivo and in vitro evidence. J Endocrinol Invest. 2019;42:715–25.
[28] Cui S, Qiao L, Yu S, Men L, Li Y, Li F, et al. The antagonist of CXCR1 and CXCR2 protects db/db mice from metabolic diseases through modulating inflammation. Am J Physiol Endocrinol Metab 2019;317:E1205-E17.
[29] Cui S, Zhu Y, Du J, Khan MN, Wang B, Wei J, et al. CXCL8 antagonist improves diabetic nephropathy in male mice with diabetes and attenuates high glucose-induced mesangial injury. Endocrinology. 2017;158:1671–84.
[30] Ranganathan P, Jayakumar C, Manicassamy S, Ramesh G. CXCR2 knockout mice are protected against DSS-colitis-induced acute kidney injury and inflammation. Am J Physiol Renal Physiol. 2013;305:F1422–7.
[31] Cheng Y, Ma XL, Wei YQ, Wei XW. Potential roles and targeted therapy of the CXCLs/CXCR2 axis in cancer and inflammatory diseases. Biochim Biophys Acta Rev Cancer. 1871;2019:289–312.
[32] Zhang Y, Ma KL, Gong YX, Wang GH, Hu ZB, Liu L, et al. Platelet microparticles mediate glomerular endothelial injury in early diabetic nephropathy. J Am Soc Nephrol. 2018;29:2671–95.