Inhibition of T-cell activation by the CTLA4-Fc Abatacept is sufficient to ameliorate proteinuric kidney disease

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Abstract

Diabetic Nephropathy (DN) remains an unmet medical challenge as its prevalence is projected to continue to increase and specific medicines for treatment remain undeveloped. Activation of the immune system, in particular T-cells, is emerging as a possible mechanism underlying DN disease progression in humans and animal models. We hypothesized that inhibition of T-cell activation will ameliorate DN. Interaction of B7-1 (CD80) on the surface of antigen presenting cells with its binding partners, CTLA4 (CD152) and CD28 on T-cells, is essential for T-cell activation. In this study we used the soluble CTLA4-Fc fusion protein Abatacept to block cell surface B7-1, preventing the cellular interaction and inhibiting T-cell activation. When Abatacept was dosed in an animal model of diabetes-induced albuminuria, it reduced albuminuria in both prevention and intervention modes. The number of T-cells infiltrating the kidneys of DN animals correlated with the degree of albuminuria and treatment with Abatacept reduced the number of renal T-cells. As B7-1 induction has been recently proposed to underlie podocyte damage in DN, Abatacept could be efficacious in DN by protecting podocytes. However, this does not appear to be the case as B7-1 was not expressed in: 1) kidneys of DN animals; 2) stimulated human podocytes in culture; or 3) glomeruli of DN patients. We conclude that Abatacept ameliorates DN by blocking systemic T-cell activation and not by interacting with podocytes.

Keywords: T-cells, albuminuria, Diabetic Nephropathy, Abatacept, CTLA4-Fc, B7-1, CD80
Introduction

Diabetic Nephropathy (DN), one of the most devastating complications of diabetes, affects one third of diabetic individuals and is the leading cause of chronic kidney disease (CKD). Progressive CKD leads to end stage renal disease (ESRD) which has become a major health problem worldwide as ESRD patients rely on dialysis or transplantation for survival (5, 31, 32). Current standard of care for DN patients comprises agents that block the Renin-Angiotensin-Aldosterone System (RAAS), like Angiotensin Receptor Blockers (ARBs) and Angiotensin Converting Enzyme inhibitors (ACEis), which provide limited protection against disease progression (19). Beyond these, specific treatments to stop/slow disease progression have not been developed. While the initiating events triggering CKD in diabetes are still under debate, a number of mechanisms involved in disease progression have been identified. Mechanical stress secondary to changes in renal hemodynamics (such as hyperfiltration and enhanced intraglomerular pressure), exaggerated production of reactive oxygen species, chronic inflammation, endothelial and tubular dysfunction and fibrosis are some of the mechanisms postulated to contribute to the pathogenesis and/or progression of DN (13, 34, 41). As there is significant interplay between these mechanisms, how efficacious intervening with each single pathway is at stopping/slowing disease progression is difficult to predict.

The immune system, in particular T-cells, is emerging as an important driver of disease in humans (29, 33). Moon et al (23) reported significant infiltration of CD3+ve T lymphocytes in the kidneys of patients with Type 2 diabetes. They reported that CD3+ve cells are the source of Interferon γ (IFNγ) and Tumour Necrosis Factor α (TNF α) in the DN kidney. In a different cohort, TNF α was found to be increased in the urine of DN patients (28) and a positive correlation between infiltration of kidney interstitial CD4+ve cells and 24-hour albuminuria was reported (23). A different study by Anand et al (1) comparing diabetic patients with and without nephropathy, found that DN subjects showed enhanced Th1 profile (increased IFN-γ, IL-2 and IL-12) suggesting systemic activation of T-cells. These findings were recently confirmed by Lei et al (18) who reported increased circulating CD8+ve T lymphocytes in plasma of type 2 diabetic patients, which was significantly associated with albuminuria. Cumulatively, these data together constitute the rationale for inhibition of T lymphocytes as a potential therapeutic approach to treat albuminuria in diabetic patients.

B7-1 (CD80) is a co-stimulatory molecule originally identified on the surface of antigen presenting cells (APC). The classical mechanism of action involves the physical
interaction with cell surface-associated counter receptors in T-cells (activating CD28 and inactivating CTLA4 (CD152), which is essential for the subsequent activation of T lymphocytes (39). The classical role that B7-1 plays within the immune system is well understood and a soluble CTLA4-Fc (Abatacept) has been developed to prevent T-cell activation and it is currently used for the treatment of rheumatoid arthritis (37, 47). A less classical mechanism of action for B7-1 has recently being suggested by Fiorina et al (12) and Yu et al (47, 52) who reported that B7-1 is expressed in podocytes during DN and that Abatacept prevents this up-regulation, leading to podocyte protection and remission of albuminuria. This less validated role of B7-1 in podocytes has been challenged by the lack of reproducibility of the findings and the poorly understood mechanistic link between B7-1 and podocyte health (14). Based on the strong evidence supporting a role for T-cells in the development and progression of DN, we hypothesized that blocking T-cell activation with Abatacept ameliorates DN by interfering with activation of T lymphocytes and not by podocyte-specific actions.
Material and Methods

Antibodies, primers and probes used

Anti-human B7-1 antibodies: Novus Biologicals 62N3G8 (Western blotting) and Abcam ab134120 (IHC). Anti-human CD68: Dako PG-M1, N1576.

B7-1 probes for in situ hybridization: human Hs-CD80, dapB (negative control) and PPIB (positive control) from Advance Cell Diagnostics.

Mouse IHC: anti-mouse CD3 from Serotec (MCA1477), anti-mouse F4/80 from Serotec (MCA497), anti Podocyn from Sigma (P0372), anti WT1 from Cambio chem (CA1026), anti-mouse collagen IV from SouthernBiotech (1340-01).

Animal studies

All experiments were conducted by Renasci Ltd, Nottingham, UK and in accordance with the project licence and Animals Scientific Procedures Act 1986. C57bl6/J (4 - 6 weeks of age, over 15g) mice were sourced from Charles River, UK, and RD12492 (60% high fat diet) from Research Diets, NJ, USA. The high-fat diet STZ-induced type 1 diabetes model was used to test the efficacy of Abatacept as it has been characterized as a model of diabetes-induced renal damage (42, 45). Upon arrival, mice used for the prevention study were placed on the high fat diet immediately while the cohort used in the intervention study was placed on normal diet (Teklad 2018) for 2 weeks and then transferred onto the high fat diet. STZ or vehicle control was administered at repeat low doses (50 mg/Kg, i.p.) for 5 consecutive days. On the morning of Day -5 a blood sample was taken from the lateral tail vein and glucose measured using the Cobas C111 analyser. Animals were allocated (on the basis of body weight and plasma glucose) to the treatment groups. Abatacept was dosed subcutaneously every second day at 10, 50 and 100 mg/Kg with treatment being initiated either 4 weeks ("prevention arm") or 11 weeks ("intervention arm") after induction of diabetes. Studies were terminated at weeks 7, 12 and 16 ("prevention arm") and 5 weeks ("intervention arm") after dosing started. Body weight was determined and recorded prior to each dose. For the intervention arm only the highest dose of Abatacept (100 mg/K. s.c) was used. As a negative control we used an IgG1 formulated in PBS. Abatacept was formulated as per supplier’s instructions, re-constituted in 10 ml water, diluted in saline and dosed in a volume of 5 ml/kg using a correction factor of 3.13 to account for excipients in the formulation. For urine collections, animals were placed into a metabolism cage with free access to food and water
for a 24 h period. To reduce evaporation, the glass urine collectors were placed in a polystyrene container (Sca-online, UK) filled with ice. Glucose, HbA1c and creatinine were determined by using the Cobas C111 analyser. Urine albumin concentrations were determined using Exocell Albuwell M EIA kits (Philadelphia, PA) according to the manufacturer’s protocol.

**Exposure levels of Abatacept**

Exposure levels of Abatacept were measured by ELISA in plasma samples obtained from the lateral tail vein. Nunc MaxiSorp ELISA plates were coated with 1μg/mL anti-id (Clone AB 550001-10) and incubated overnight at 2-8°C. Plates are washed with 1X ELISA wash buffer after incubation and blocked with I Block Buffer. After washing step, standards, controls, and samples are plated and incubated for 1 hour ± 10 minutes, with shaking at room temperature. Unbound materials are removed after incubation by washing with 1X ELISA wash buffer. Biotinylated anti-id (Clone AB 550033-6) is added and allowed to react for approximately 1 hour ± 10 minutes at RT. Then streptavidin HRP is added to incubate for approximately 60 minutes. Excess enzyme conjugate is removed by washing and TMB substrate is added to the wells. The intensity of substrate colour is proportional to the amount of Abatacept present in the sample. Enzyme reaction is quenched by addition of 4N sulfuric acid (H2SO4). Absorbance is measured at 450 nm. Antibody concentrations are interpolated from the standard curve using the SOFTmax PRO GxP 5-parameter fit program. Minimum required dilution of the assay is 1:50.

**Cultured immortalized human podocytes**

Conditionally immortalized human podocytes, kindly donated by Richard J Coward, were used as previously described (35). Once immortalized, podocytes were maintained in culture at 33°C. Experiments on differentiated podocytes were performed after silencing of the temperature sensitive simian virus 40 large T antigen (SV40 LTAg) at 37°C for at least 14 days, to allow them to differentiate and for experiments, cells were used in passages between 5 and 20. This is important, as in vivo podocytes are terminally differentiated cells. Cells were cultured in RPMI-1640 and supplemented with 10% (vol/vol) bovine fetal serum (FBS). Once differentiated, human podocytes were deprived of FBS for at least 8 h prior to stimulation and cells were treated with bacterial lipopolysaccharides (LPS) at doses ranging from 20 to 50 µg/mL for 24 h; High Glucose (HG) 30 mM for different time points (between 3 days and 14 days) with mannitol used as osmotic control; TGFβ 5 ng/mL for 24, 48 and 72
hrs and Palmitic Acid (PA) at 100, 500 and 750 μM for 24 hr. At the end of the treatment period, podocytes were lysed in Tryzol for downstream analysis of gene expression. When podocytes were used for protein detection by western blotting, they were homogenized in lysis buffer containing a protease and phosphatase inhibitor cocktail.

**Immunodetection of B7-1 in human podocytes (Western blotting)**

For each sample, 25 μg total protein was resolved by electrophoresis using 4–20% precast polyacrylamide gels under denaturing conditions. Proteins were transferred to nitrocellulose membranes and incubated with PBS-blocking solution (Li-Cor Biosciences) for 1 hr following incubation with 1:500 dilution anti-CD80 antibody (Novus Biologicals 62N3G8) and 1:5,000 secondary tagged antibody (Li-Cor) for 1 hr at room temperature and following Li-Cor PBS protocol. Imaging was carried out using Odyssey technology (Li-Cor). Fluorescent detection was performed after incubating with the corresponding secondary antibodies.

Antibody validation: a panel of commercially available anti-human B7-1/CD80 antibodies were tested: (a) R&D Systems catalogue number AF140, (b) Biolegend 2D10 catalogue number 305202, (c) Novus Biologicals 62N3G8 catalogue number NBP2-25255 and, (d) Novus Biologicals 2E5 catalogue number NBP2-02905. As loading control for cellular homogenates, the anti-human GAPDH antibody from Sigma (catalogue number G9545) was used. As positive control we used recombinant human B7-1 and Rajji and Daudi cell homogenates. As negative controls we used mouse recombinant B7-1 and rat fibroblast homogenates.

**RNA extraction and quantitative real-time polymerase chain reaction (qPCR).**

For podocytes, total RNA was extracted using TRI Reagent (Sigma). 1 μg of RNA was reverse transcribed with High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). qPCR was performed using the Sybr Green method and d(N)6 random hexamer with the primers for human B7-1 previously reported\(^{17}\). RNA B7-1 was expressed relative to β-actin expression.

For kidney tissues, total mRNA was extracted using a magnetic bead-based semi-automated method (ThermoFisher Scientific). After cDNA synthesis, gene expression (relative to gapdh) was measured using TaqMan probes (Applied Biosystems) and fold change in expression calculated using the ΔΔCt method.
**Immunohistochemistry**

Fixed 3-4 μm sections of mouse and human kidneys were used and staining performed either on the Dako Flatbed autostainer (CD3, F4/80, B7-1) or the Ventana Ultra (Podocyn, WT1, Collagen IV). Analysis was done by using the Aperio ePathology Digital Image Analysis Software, Definiens Tissue Studio or Visiopharm.

Immunodetection of B7-1 was performed in 20 human kidney biopsies collected under consent at the Sahlgrenska University in Gothenburg, Sweden (10 DN and 10 controls). Patient characteristics were as follows:

<table>
<thead>
<tr>
<th>Group</th>
<th>Gender</th>
<th>Age average ± SD</th>
<th>Serum creatinine μmol/L average ± SD</th>
<th>albuminuria g/24h average ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6m/4f</td>
<td>53.5 ± 14.2 (Range: 38 - 87)</td>
<td>ND*</td>
<td>ND*</td>
</tr>
<tr>
<td>DN</td>
<td>7m/3f</td>
<td>56.4 ± 9.1 (Range: 41-67)</td>
<td>204 ± 97 (Range: 102 – 412)</td>
<td>4.1 ± 3.2 (Range: 1.3 – 10.0)</td>
</tr>
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*ND = not determined. The control group consist of kidney transplant donors, who are checked to have normal creatinine and no albuminuria.

The EnVision™ Flex high pH (Link) detection kit (Dako K8000, Copenhagen, Denmark) was used. Consecutive 4-μm series of paraffin sections underwent antigen retrieval in Tris/EDTA buffer, pH 9 (Dako K8004) by microwave oven heating and endogenous peroxidase activity was blocked by immersion in peroxidase-blocking solution (Dako K8000) for 5 minutes at RT. Immunostaining was performed in a computer-assisted Autostainer Plus processor (Dako). Incubation time for the primary antibodies was 30 minutes at RT, terminated by repeated washings, followed by incubation with a dextran polymer conjugated with secondary antibodies and horseradish peroxidase (HRP) for another 30 minutes. Slides were transferred to fresh hydrogen peroxide plus 3-3-diaminobenzidine tetra hydrochloride (DAB) solutions for 4 minutes. Finally, slides were stained with Mayer’s haematoxylin and permanently mounted under cover slips. Omitting or replacing the primary antibody with irrelevant antibodies produced negative controls. Optimal primary antibody dilutions were defined by staining normal tonsils using serial dilutions of the antibodies. The following antibodies and final concentration were used; CD68 1:100 (PG-M1, Dako, N1576), B7-1/CD80 1:300 (ab134120, Abcam, Cambridge, UK).
B7-1 +ve cells were counted manually. Two pathologists (JM, MS) made independent calculations and the average score was recorded from each patient. CD68 +ve cells were counted using automated image analysis. Immuno-stained tissue sections were digitized using a slide scanner (Aperio) fitted with a 20x, air objective yielding images with 2.027 pixels/µm. The resulting virtual slides were imported into Visiopharm Integrator System software (version 5.0.1, Visiopharm, Hørsholm, Denmark). Kidney tissue cores on each slide were identified and central analysis regions were created by excluding a 30 µm edge band (to avoid morphological and staining artifacts). The total number of cells in the measurement area was determined and classified cell populations are reported as a percentage of the total number for that slide (on average more than 30,000 cells were evaluated per slide).

**In situ hybridization**

In situ hybridization was performed in fixed 3 µm sections using RNAScope technology following manufacturer’s instructions (Advance Cell Diagnostics). The following human probes were used: Hs-CD80, dapB (negative control) and PPIB (positive control). Manual InSitu Hybridisation was performed according to the RNAScope instructions manual (reference http://www.acdbio.com/technical-support/user-manuals). FFPE kidney sections were prepared and pre-treated as recommended for the tissue types. The target and control probes were hybridized for 2 hours at 40°C followed by each amplification hybridization stage. The signal was detected using DAB substrate, counterstained with Haematoxylin, dehydrated, mounted and analysed using the recommended semi quantitative scoring guidelines. Validation of the probes was done using human tonsil tissue as a positive control.

**Statistical analysis**

Results are expressed as mean ± standard error of the mean. For the animal studies, all statistical analysis was performed by a Statistician using SAS® 9.3. Each dose of Abatacept was compared to vehicle by Williams’ test. P<0.05 was the level accepted for statistical difference. The chance of a false positive is 5% for each compound for each time. For the *ex vivo* and *in vitro* studies, statistical analysis was performed using GraphPad Prism 6. Differences between groups were analysed by ANOVA with subsequent multiple comparison test correction (Bonferroni). All tests were carried out as two sided tests. The human histology data was analysed by unpaired t-test. The statistical significance of the correlation between albuminuria and CD3 + cells in the kidney was interrogated by calculating the Pearson Correlation Coefficient.
Results

Abatacept administration reduces albuminuria in diabetic mice

To test the ability of the soluble CTLA4-Fc (Abatacept) to lower albuminuria, we used the 60% high fat-diet mouse Streptozotocin (STZ)-induced model of diabetes.

In both prevention and intervention protocols, Abatacept treatment reduced albuminuria (Figure 1). In the prevention arm, in which Abatacept treatment started 4 weeks after diabetes was induced, all three doses were efficacious at reducing urinary albumin/creatinine excretion after 7, 12 and 16 days of dosing (Figure 1, panel A) with some groups exhibiting complete attenuation of albuminuria. Similarly, in the intervention arm, in which Abatacept treatment started 11 weeks after diabetes was established, complete remission of albuminuria was achieved after 5 weeks of dosing with 100 mg/Kg Abatacept (Figure 1, panel B).

In the STZ-treated group, glomerular filtration rate (GFR) was found increased but treatment with Abatacept did not reduce hyper-filtration (Figure 2). In addition, plasma glucose or HbA1c levels did not change in any of the groups (Tables 1 and 2). As reported previously (21), the STZ treatment significantly affected body weight gain in vehicle-treated animals being 29.3 ± 0.4 g versus 46.5 ± 0.9 g in the non-STZ group at the end of the study (p< 0.001, n=13 and 12 respectively). Treatment with Abatacept did not affect body weight among groups (Table 3). No differences were found in kidney weights at the end of the study (Table 3).

The exposure levels of Abatacept were verified in plasma. In the prevention groups, at week 12 of dosing, Abatacept plasma levels were 495 ± 24, 1,035 ± 58 and 1,561 ± 67 μg/mL, in animals dosed with 10, 50 and 100 mg/Kg respectively. Similarly, at week 16 of dosing, exposure levels were 398 ± 24, 1,011 ± 46 and 1,426 ± 89 μg/mL, in animals dosed with 10, 50 and 100 mg/Kg respectively. In the intervention arm, exposure levels of CTL4-Fc were 1,521± 62 μg/mL at week 5 after dosing started with 100 mg/Kg/day.

Abatacept does not affect glomerular hyperfiltration in diabetic mice

Gloremular filtration rate (calculated as clearance of creatinine) was elevated in STZ-induced diabetic mice at week 20 (STZ-IgG group). The glomerular hyper-filtration was not affected by Abatacept at any given dose or regimen (Figure 2).
Abatacept administration reduces renal T-cell infiltration in diabetic mice

In the renal cortex, the number of CD3+ve cells in the STZ-induced diabetes group at the end of the study (20 weeks after diabetes induction), was increased and this increase was completely inhibited by administration of 50 and 100 mg/Kg/day Abatacept for 16 weeks (prevention arm) (Figure 3, Panel A, left panel). Similarly, in the intervention arm, the number of CD3+ve cells infiltrating the kidney cortex was increased after 11 weeks of diabetes induction and was reduced to the control values after treatment with 100 mg/Kg/day Abatacept for 5 weeks (Figure 3, Panel A, right panel). Most CD3+ve cells located in the peri-glomerular space (Figure 3, panel B). Similar results were found when the kidney medulla was included in the quantification. Moreover, the number of T-cells in the renal cortex positively correlated with the degree of albuminuria in the diabetic mice 20 weeks after diabetes induction (Figure 3, panel C).

Renal F4/80+cells were not increased in diabetic mice

The number of F4/80+ve cells in the kidney cortex was unchanged in of STZ-mice compared to the Non-STZ control group at study termination (20 weeks after diabetes induction). Abatacept treatment (all doses and regimens) did not affect the number of F4/80+ve cells infiltrating the kidney of diabetic animals. Similar results were found when the kidney medulla was included it in the analysis. (Figure 4, Panel A)

Collagen IV was increased in diabetic kidneys

Cortical collagen IV was increased in of STZ-mice compared to the Non-STZ control group at study termination (20 weeks after diabetes induction). This mild increase in collagen IV remained elevated in diabetic mice treated with the lower dose of Abatacept (50 mg/Kg/day) for 16 weeks but was no longer significant in animals treated with 50 and 100 mg/Kg/day Abatacept (Figure 4, A).

Neither podocyte number nor B7-1 expression was affected in the STZ-induced diabetes animals.

We next performed immunostaining for the podocyte-specific proteins WT1 and Podocin at the end of the study (20 weeks after induction of diabetes, prevention arm). As depicted in Figure 4 panel B, the STZ treated animals did not display signs of podocyte loss as the percent of WT1 positive nuclei in the glomerular area was preserved compared to the
non-STZ group. Similarly, Podocin staining was not different. Treatment with Abatacept (50 mg/Kg/day) did not modify podocyte number (Figure 4, Panel B).

For Abatacept to have a direct protective effect in podocytes requires that its binding partner B7-1 is expressed in these cells as reported by Fiorina et al (12). In the non-STZ group, the mRNA expression of B7-1 in the renal cortex was $2.8 \pm 0.6 \times 10^{-6}$ times the levels of GAPDH (calculated as $2^{-\Delta\Delta CT}$), indicating a very low level of expression of B7-1 at the mRNA level. B7-1 was not induced in the kidney of diabetic mice after 20 weeks and treatment with Abatacept did not affect B7-1 mRNA expression (Figure 4, Panel C).

**B7-1 is not induced in stressed human podocytes in culture**

Because we did not detect expression of B7-1 in the mouse kidney, we then investigated whether expression of B7-1 could be induced in immortalized human podocytes in culture as others have reported (6, 12, 40). When assessed by qPCR using previously reported Sybergreen primers (12), we found that neither treatment with palmitic acid (PA, 100-750 μM for 24 hr), transforming growth factor β (TGF β 5 ng/mL, for 24-72 hr), bacterial lipopolysaccharides (LPS, 20 and 50 μg/mL for 24 hr) nor high glucose (HG, 30 mM for 3-14 days) increased B7-1 mRNA expression (Table 4).

B7-1 protein expression was measured by Western blotting. To find a suitable antibody that specifically recognizes human B7-1, we tested a panel of commercial antibodies using cell lines and recombinant B7-1 as positive controls and human kidney fibroblast homogenates as a negative control. We identified an antibody from Novus Biologies (Novus 62N3G8) that recognized a diffuse, 65-kDa band in T-cells consistent with the glycosylated state of B7-1 and its predicted MW of 65-kDa (Figure 5, panel A). Using this antibody we failed to detect B7-1 in human immortalized podocytes under either basal or LPS- or HG-stimulated conditions (Figure 5, panel B).

**B7-1 (CD80) is not expressed in human kidneys from diabetic nephropathy individuals**

Finally we conducted immunostaining of human kidneys to investigate expression B7-1. Using the Rabbit monoclonal anti human CD80 (Abcam, ab134120) antibody, we did not detect any B7-1 staining in the glomerular compartment of either controls or diabetic nephropathy patients. However, a small number of B7-1 positive cells were detected in the interstitial space (Figure 6, panel A). The antibody used for the immunostaining was validated by using human tonsil tissue as the positive control (Figure 6, panel A). The isotype
control antibody showed no staining. The number of B7-1 positive cells was not significantly different in DN patients compared to controls (Figure 6, panel B). In contrast to the STZ-animal model, the number of total macrophages (CD68 +ve cells) infiltrating the kidneys of DN patients was significantly increased as shown in the table in Figure 6.

We have also performed in situ hybridization to investigate if B7-1 mRNA was detected in tissues from four CKD patients. To validate the probes tonsil tissue was again used (Figure 6, C). Confirming our IHC data, B7-1 mRNA was again undetected in the glomeruli of kidney biopsies from either control or DN patients with scattered staining in the interstitial space (Figure 6, C).
Discussion

In this study we found that blocking T-cell activation is sufficient to achieve reduction in albuminuria in the STZ-induced DN mouse model. For many years the role of immune cells in the development/maintenance of DN has been investigated with particular emphasis on macrophages (3, 17, 46, 49). It is known that macrophages infiltrate the kidneys of DN patients (16, 48) and it is postulated that they activate a cascade of pro-fibrotic signalling processes that contribute to sclerosis and scarring of the kidneys (4, 11, 22). In fact, this constitutes the rationale for the mechanism of action of drugs in development for DN (25, 36, 44). Less attention has been placed on T-cells and even though publications demonstrate that T-cells also infiltrate the kidneys of these patients (51), studies showing a direct impact of T-cell suppression on DN had not yet being done to the best of our knowledge.

Our data demonstrate that blocking T-cell activation systemically reduces the number of T-cells infiltrating the kidneys and that these correlated with a significant attenuation of renal damage as measured by albuminuria. The reduction in albuminuria was independent of GFR since the treatment did not affect the diabetes-induced glomerular hyperfiltration (Figure 2). While there was clear dose proportionality between dosed and plasma levels of Abatacept, a dose-related effect on albuminuria was not apparent suggesting that the 10 mg/Kg dose is maximally renal protective. The degree of renal protection we found with Abatacept is impressive, in particular for the intervention arm in which Abatacept dosing started 11 weeks after diabetes was established. Treating these animals with Abatacept for 5 weeks completely normalized albuminuria (Figure 1, B). The intervention arm data shows that Abatacept is effective when treatment is started even when T-cell infiltration and albuminuria are already elevated. Moreover, the importance of renal T-cells was evident by the positive correlation found between renal CD3 +ve cells and the degree of albuminuria in diabetic animals. In addition, Abatacept reduced renal T-cells and kidney damage suggesting that during DN, there is activation of T-cells that invade the kidney and participate in renal damage.

In this study the number of T-cells infiltrating the kidneys was assessed by CD3 + staining. Evidence in the literature shows that in this model of STZ-induced albuminuria, the number of CD3-derived CD4+ and CD8+ cells increases proportionally mirroring the CD3 data (10, 23, 43). For instance, Moon et al (23) looked at the inflammatory infiltrate in the kidney of STZ-mice and found an increase in CD3+, CD4+ and CD8+ cells. Interferon-Ƴ and
tumor necrosis factor-α mRNA expression was also elevated in diabetic mouse kidneys compared with controls. Further, flow cytometric analysis of kidney-derived mononuclear cells showed increased production of IFN-Ɣ and TNF-α by CD3+ T cells. Suggesting that helper and cytotoxic T-cells are important players driving disease progression in this model. The participation of T-cells in human DN has recently been demonstrated by a significant correlation between kidney T-cells and albuminuria in type II diabetics (23). Moreover these authors showed significant number of CD4 and CD8 positive cells infiltrating the human DN kidneys. Even though a thorough characterization of the immune cell populations infiltrating the kidney during human DN has not been reported, understanding the immune cell phenotype in this condition might aid in the development of more specific approaches to treat this disease. The specific signalling events underlying the migration and homing of T-cells to the kidney during diabetic nephropathy are still unknown, but adhesion molecules and chemokines are likely to play a role (9, 15, 24, 38). Animal studies indicate that the adhesion molecule ICAM-1 (CD54) is upregulated in the glomeruli of STZ-induced rodent diabetes and that kidney T-cell infiltration can be reduced by an ICAM-1 monoclonal antibody (43). Moreover, ICAM-1 deficient db/db mice have decreased homing of CD4+ T cells into diabetic kidneys and reduced renal damage (8). In addition, the chemotactic cytokine RANTES (CCL5), has been shown to direct homing of CD4+ T-cells in the kidneys of different renal disease models (2, 26, 30). Although the involvement of adhesion molecules or cytokines in the infiltration of T cells in human DN remains to be demonstrated, human studies finding that the RANTES promoter genotype is associated with diabetic nephropathy in type 2 diabetic subjects (27) suggest that these might play an important role in disease progression.

The participation of renal macrophages has been suggested to be important for disease progression in humans. In our study we found that the number F4/80+cells in the kidney of diabetic animals is not elevated at the end of the study (20 weeks of diabetes). This suggests that in our animal model, macrophages might play a negligible role at driving renal damage and that T-cell activation could be sufficient to drive renal injury independent of macrophages. This is different to our observation in humans in which a significant amount of macrophages are present in DN kidneys. The differences possible arise from the fact the animal and human observations are done at different stages of disease progression. In humans, activated T-cells within the kidney could cause damage directly through cytotoxic effects and indirectly by the recruitment and activation of macrophages. Pro-inflammatory
cytokines released by T-cells can stimulate the release of Colony Stimulating Factor-1 and Monocyte Chemoattractant Protein-1 from mesangial cells leading to macrophage infiltration and activation (7). Whether or not inhibition of T cell activation impairs macrophage infiltration/activation in human DN remains to be investigated and therapies targeting T-cell might prove beneficial in treating DN. In this study we found a significant impact of Abatacept on renal T-cell infiltration. However, whether this is due to a direct effect on lymphocyte migration or if it is due to systemic reduction in circulating T-cells is currently unknown. Other reports have shown a systemic activation of T-cells in DN (50).

To explore the mechanisms of the beneficial effects of CTAL4-Fc in this model, we measured fibrosis and podocyte number. The percent area of collagen IV immunostaining increased 20 weeks after diabetes started compared to the non-STZ group. In the top two doses of Abatacept-treated animals, the increase in collagen IV was no longer significant but statistically significant differences between STZ and Abatacept treatment groups were not detected. Although it is possible that this study did not have enough power to detect a significant improvement on this endpoint by Abatacept, the small degree of collagen IV upregulation in STZ-mice suggest that fibrosis is not a significant component of the mechanisms driving renal damage in this model. In addition, we did not detect reductions in podocyte numbers (WT1 staining) or area (podocin stain) which suggest that in this model, podocyte loss is not the main mechanism mediating albuminuria. Podocyte B7-1 has been postulated to be induced in human DN and to mechanistically participate in disease progression (12, 40). We ruled out the participation of B7-1 in podocytes in driving disease in this model as we did not detect B7-1 mRNA in the mouse kidneys (Fig 3). Unlike the findings from Fiorina et al (10), we did not detect induction of B7-1 in the mouse kidney cortex by real-time PCR using TaqMan probes. The lack of up-regulation of kidney B7-1 in the STZ mouse further supports our conclusion that the efficacy of Abatacept in this DN model is not due to a direct effect on podocytes as Abatacept is not expected to directly interact with these cells that lack its cellular B7-1 binding partner. In addition, we systematically assessed B7-1 protein and mRNA expression in human samples. First, we analysed a total of 10 kidney biopsies of DN patients. To validate the antibodies we used human tonsil samples and fixed Raji cells. Using formalin fixed paraffin embedded (FFPE) tissue we did not detect B7-1 staining inside the glomerulus of any patient analysed. A few cells stained positive in the interstitial area indicating the presence of APC as previously reported. The fact that we did detect positive cells outside the glomeruli using FFPE tissue,
further validates the immunohistochemistry protocol utilized, as it has been suggested that frozen samples need to be used to preserve B7-1 antigens. Using in situ hybridization of human kidneys again B7-1 mRNA was detected in the extraglomerular space but no signal was present inside the glomeruli. Our data demonstrate the lack of B7-1 mRNA and protein in the glomeruli of human kidney biopsies and challenge previous findings suggesting up-regulation of B7-1 in the glomeruli of DN patients (12, 52). Our findings are consistent with recent findings reporting absence of B7-1 in human podocytes (14).

As others have reported induction of B7-1 in vitro (18, 47) we used differentiated human immortalized podocytes stimulated with different insults to explore B7-1 expression. Using the method reported by Fiorina et al (12) B7-1 mRNA was not induced in podocytes treated with palmitic acid, TGFβ, LPS or HG (Figure 5). The basal C_T values detected by Cyber green-based amplification were 30 for B7-1 and 18 for β-actin (housekeeping) suggesting relatively low levels of B7-1 expression. In addition, we were not able to detect amplification when using TaqMan probes suggesting possible poor specificity of cyber green-based techniques. Moreover, using a validated antibody for Western blotting-based detection, B7-1 protein was undetected under both, basal and stimulated conditions. We conclude that B7-1 is not expressed in human and mouse podocytes either under normal or diseased conditions and that the protective effect of Abatacept during DN is not due to its direct interaction with podocytes. These results indicating the absence of B7-1 in podocytes from humans and mouse were recently validated by a different group reporting very similar findings in a different patient cohort and they contradict the studies of Fiorina et al (12) and Yu et al (52). Our data demonstrate the absence of B7-1 in mouse and human podocytes and do not support its involvement in podocyte dysfunction during experimental DN in the STZ mouse. Our data also contradict previous reports demonstrating up-regulation of B7-1 in stimulated human podocytes (10, 34). The explanation for the differences between our reports and the data of Fiorina et al. is not completely understood. However, part of the differences may rely on the fact that Fiorina et al used an antibody that we invalidated (R&D140, Figure 5) to detect B7-1 expression in their podocyte studies.

The mechanism of action of Abatacept has clearly been demonstrated as a potent inhibitor of T-cell activation (20) and the lack of B7-1 expression in the animal model and human biopsies suggest that the site of action of Abatacept is not the podocyte. The final pathogenic mechanism/s targeted by T-cells during DN is unknown but there are many
possibilities that remain to be studied. As for the mechanism of action driving albuminuria reduction, it is possible that protection of the glomerular filtration barrier that cannot be detected by conventional IHC, is the explanation however, these studies are beyond the scope of the present study. In addition, direct protection in endothelial cells and proximal tubules may account for the amelioration of albuminuria exhibited by Abatacept. Our data show that renal collagen IV is increased in STZ-treated mice but the magnitude of this increase is rather small. As the STZ-mice does not develop very impressive fibrosis, perhaps the effects of Abatacept in humans would yield anti-fibrotic effects. We found that collagen IV and CD3+ cells were increased before intervention was initiated. After 24 days of dosing with Abatacept the renal T-cell number was reduced without clear changes in collagen IV. It is important to highlight the fact that the animal model we used does not develop massive renal fibrosis and therefore, the structural changes induced by T-cell inhibition in humans are difficult to predict. But even if T-cell inhibition does not result in reversal of structural changes, since Abatacept reverses albuminuria experimentally, this treatment has the potential to “slow”, and even “stop”, renal damage and disease progression in DN patients preventing them from reaching end stage renal disease.

In conclusion, we demonstrated that blocking T-cell activation ameliorates albuminuria in a proteinuric diabetic model and that the target cells responsible for this effect is not the podocyte but rather the result of the systemic inhibition of T-cell activation. Thus, targeting T-cells might be a novel strategy to treat proteinuric kidney diseases with the potential to slow or stop disease progression.

Disclosures

There are no sources in the current document. The authors have declared that no conflict of interest exists.
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Figure Captions

Figure 1: Effect of the Abatacept on urinary albumin/creatinine ratio. At all doses, Abatacept was efficacious at attenuating albuminuria when administered every 2 days (s.c) in both, prevention (A) and intervention (B) modes.

Figure 2: Effect of Abatacept on glomerular filtration rate (GFR) calculated as clearance of creatinine at the end of the study (20 weeks since diabetes induction, 16 weeks of dosing). Abatacept had no effect on STZ-induced glomerular hyper-filtration. n= 9-13 per group.

Figure 3: Effect of the Abatacept (across different regimens) on the number of lymphocytes (CD3+ve cells) infiltrating the kidney cortex in STZ-induced diabetes animals (A). Representative images of peri-glomerular localization of CD3+ cells of an STZ-diabetic mice 20 weeks after diabetes induction (B). Positive correlation between the number of lymphocytes present in the renal cortex versus the degree of albuminuria in STZ-induced diabetic nephropathy animals (C). n= 9-13 per group.

Figure 4: (A) Effect of the Abatacept (across different regimens) on the number of macrophages (F4/80+ve cells) infiltrating the kidney; and collagen IV. (B) Effect of Abatacept (50 mg/kg) on glomerular size; the number of podocytes by WT1 stained cells in the glomeruli; the podocyte area (podocin +ve cells) and renal cortical mRNA expression of B7-1 in STZ-induced diabetes animals. n= 9-13 per group

Figure 5: (A) Validation of anti-human B7-1 antibodies. Of all 4 commercial antibodies tested, we identified an antibody from Novus Biologics (Novus 62N3G8) that recognized a diffuse, 65-kDa band in T-cells consistent with the glycosylated estate of B7-1 and its predicted MW of 65 kDa. This antibody also recognized recombinant human B7-1 although yielding a slightly lower MW. This could be due to intrinsic modifications of the protein introduced to facilitate the purification process. (B) absence of B7-1 protein in podocytes with and without LPS and HG treatment. Conditionally immortalized human podocytes were differentiated at 37°C for at least 14 days and were used for experiments in passages between 5 and 20.

Figure 6: B7-1 was not found expressed in podocytes of human biopsies. (A) Summary data indicating the number of B7-1 positive cells and macrophages in kidneys of control and diabetic nephropathy (DN) patients using the ab134120 antibody (n=10 DN and 10 controls). (B) The left picture is a representative example of B7.1 staining in a DN kidney sample. The
right panel shows staining of B7-1 in human tonsil (positive control). (C) Detection of B7.1
by in situ hybridization, n=4 DN and 4 controls.
### Table 1: Effect of the different administration regimens of Abatacept in plasma glucose and HbA1c in the prevention arm. *: p<0.05 vs STZ groups

<table>
<thead>
<tr>
<th>Groups (prevention)</th>
<th>Weeks after drug dosing</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7</td>
<td>12</td>
<td>16</td>
<td>7</td>
<td>12</td>
<td>16</td>
</tr>
<tr>
<td>plasma glucose (mM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-STZ</td>
<td>12.9 ± 0.4*</td>
<td>13.5 ± 0.3*</td>
<td>14.9 ± 0.9*</td>
<td>4.8 ± 0.1*</td>
<td>4.7 ± 0.1*</td>
<td>5.4 ± 0.1*</td>
</tr>
<tr>
<td>STZ-IgG1 10 mg/Kg/day</td>
<td>32.6 ± 0.7</td>
<td>33.3 ± 0.5</td>
<td>37.0 ± 1.0</td>
<td>7.9 ± 0.1</td>
<td>8.2 ± 0.1</td>
<td>8.7 ± 0.1</td>
</tr>
<tr>
<td>STZ- Abatacept 10 mg/kg</td>
<td>31.3 ± 0.8</td>
<td>34.2 ± 0.9</td>
<td>36.5 ± 1.7</td>
<td>7.6 ± 0.2</td>
<td>8.1 ± 0.2</td>
<td>8.6 ± 0.2</td>
</tr>
<tr>
<td>STZ- Abatacept 50 mg/kg</td>
<td>31.5 ± 1.0</td>
<td>32.9 ± 1.0</td>
<td>35.7 ± 1.0</td>
<td>7.8 ± 0.1</td>
<td>8.2 ± 0.1</td>
<td>8.7 ± 0.2</td>
</tr>
<tr>
<td>STZ- Abatacept 100 mg/kg</td>
<td>31.7 ± 0.8</td>
<td>33.8 ± 0.9</td>
<td>35.1 ± 2.0</td>
<td>7.8 ± 0.1</td>
<td>8.4 ± 0.1</td>
<td>8.8 ± 0.1</td>
</tr>
</tbody>
</table>
Table 2: Effect of Abatacept in plasma glucose and HbA1c in the intervention arm. * : p<0.05 vs STZ groups.

Table 3: Effect of STZ and Abatacept on body and kidney weights at the end of the prevention and intervention studies. * : p <0.001 vs all other groups.
Table 4: Lack of induction of B7-1 mRNA in cultured podocytes under different insults. Conditionally immortalized human podocytes were differentiated at 37°C for at least 14 days and were used for experiments in passages between 5 and 20. Fold change is calculated related to vehicle control within the same experiment. No significant differences were found between groups. n= 5-8. Average Ct values: 30 (B7-1) and 18 (housekeeping β-actin).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>B7-1 mRNA [fold change from control]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic acid 100 mM 24 hr</td>
<td>2.2 ± 0.8</td>
</tr>
<tr>
<td>Palmitic acid 500 mM 24 hr</td>
<td>2.3 ± 0.9</td>
</tr>
<tr>
<td>Palmitic acid 750 mM 24 hr</td>
<td>3.2 ± 0.7</td>
</tr>
<tr>
<td>TGFβ (5ng/mL) 24 hr</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>TGFβ (5ng/mL) 48 hr</td>
<td>-0.5 ± 0.2</td>
</tr>
<tr>
<td>TGFβ (5ng/mL) 72 hr</td>
<td>-1.2 ± 0.3</td>
</tr>
<tr>
<td>LipopolySacharides 20 mg/mL 24 hr</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td>LipopolySacharides 50 mg/mL 24 hr</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>High Glucose (30 mM) 3 days</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>High Glucose (30 mM) 5 days</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>High Glucose (30 mM) 10 days</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>High Glucose (30 mM) 14 days</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>Mannitol (30 mM) 10 days</td>
<td>0.8 ± 0.2</td>
</tr>
</tbody>
</table>
A  Prevention: dosing started 4 weeks after diabetes induction

![Bar chart showing urine Alb/Creat (µg/µmol) over weeks after drugs dosing started]

* *p<0.05 vs STZ vehicle at same time point (white bar)

B  Intervention: dosing started 11 weeks after diabetes induction

![Bar chart showing urine Alb/Creat (µg/µmol) before and after dosing]

* *p<0.05 vs STZ vehicle at same time point (white bar)

Figure 1
Figure 2

* : $p < 0.05$ vs Non-STZ
Prevention: dosing started 4 weeks after diabetes induction

Intervention: dosing started 11 weeks after diabetes induction

* : $p < 0.001$ vs Non-STZ. # : $p < 0.05$ vs STZ-IgG control (ANOVA outcome $p < 0.0001$)

CD3 staining in STZ-induced diabetes

Figure 3
A

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>F4/80 [n²/mm²]</th>
<th>Cortical Collagen IV [% of area]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-STZ</td>
<td>75.4 ± 15.6</td>
<td>7.8 ± 0.8</td>
</tr>
<tr>
<td>STZ-IgG-50 mg/Kg</td>
<td>74.1 ± 6.6</td>
<td>11.9 ± 1.1 *</td>
</tr>
<tr>
<td>STZ-Abatacept 50 mg/kg (prevention arm)</td>
<td>101.9 ± 17.8</td>
<td>11.2 ± 0.7 *</td>
</tr>
<tr>
<td>STZ-Abatacept 100 mg/kg (prevention arm)</td>
<td>101.6 ± 11.6</td>
<td>8.4 ± 0.5</td>
</tr>
<tr>
<td>STZ-Abatacept 100 mg/kg (intervention arm)</td>
<td>74.9 ± 10.8</td>
<td>11.2 ± 1.2</td>
</tr>
</tbody>
</table>

* p<0.05 vs Non-STZ. Collagen IV ANOVA outcome: p < 0.01

B

<table>
<thead>
<tr>
<th>Experimental Group (20 weeks after diabetes induction)</th>
<th>Mean glomerular area [µm²]</th>
<th>WT1+ cells [%]</th>
<th>Mean podocin area [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-STZ</td>
<td>4247 ± 101</td>
<td>32.6 ± 2</td>
<td>35 ± 3</td>
</tr>
<tr>
<td>STZ-IgG-50 mg/Kg for 16 weeks (prevention arm)</td>
<td>4477 ± 54</td>
<td>33.4 ± 3</td>
<td>36 ± 3</td>
</tr>
<tr>
<td>STZ-Abatacept 50 mg/kg for 16 weeks (prevention arm)</td>
<td>4214 ± 111</td>
<td>34.1 ± 1</td>
<td>36 ± 2</td>
</tr>
</tbody>
</table>

Figure 4
A

Anti human B7-1 (CD80) antibodies

Green: GAPDH
RED: B7-1 (CD80)

<table>
<thead>
<tr>
<th>Lines:</th>
<th>Expected MW:</th>
</tr>
</thead>
<tbody>
<tr>
<td>1- NRK49F (rat fibroblasts) 30 ug</td>
<td>GAPDH 37 kDa</td>
</tr>
<tr>
<td>2- m CD80 recombinant 20 ng</td>
<td>B7-1 (CD80) 65 kDa</td>
</tr>
<tr>
<td>3- h CD80 recombinant 20 ng</td>
<td></td>
</tr>
<tr>
<td>4- Raji cells vehicle 30 ug</td>
<td></td>
</tr>
<tr>
<td>5- Raji cells + PHA 30 ug</td>
<td></td>
</tr>
<tr>
<td>6- Daudi cells vehicle 30 ug</td>
<td></td>
</tr>
<tr>
<td>7- Daudi cells + PHA 30 ug</td>
<td></td>
</tr>
</tbody>
</table>

B

B7-1 detection using Novus 62N3G8 antibody (human cultured podocytes)

LPS 20 or 50 µg/mL
Glucose or mannitol 30 mM

Figure 5
A. Validation of anti-B7-1 antibody (ab134120)

B-1 staining (DN)  Human tonsil (+ ctrol)

B. Macrophage and B7-1 positive staining in DN biopsies

<table>
<thead>
<tr>
<th>Groups</th>
<th>Pan-MØ (CD68 +)</th>
<th>B7-1+ (antibody: ab134120)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=10)</td>
<td>332 ± 36</td>
<td>0.10 ± 0.06</td>
</tr>
<tr>
<td>Diabetic Nephropathy (n=10)</td>
<td>1906 ± 142*</td>
<td>0.40 ± 0.09</td>
</tr>
</tbody>
</table>

Results are shown as number of positive cells/mm² examined area, ± SE.
*: p<0.05 vs control.

C. B7-1 mRNA detection by In situ hybridization in DN kidneys

Human tonsil

Positive B7.1 probe  Negative ctrol probe

DN stage 2  glomerulus  DN stage 3  Tubulo-interstitium

DN stage 3  Tubulo-interstitium

Figure 6