Antibody blockade of *c-fms* suppresses the progression of inflammation and injury in early diabetic nephropathy in obese *db/db* mice

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Abstract

Aims/hypothesis Macrophage-mediated renal injury plays an important role in the development of diabetic nephropathy. Colony-stimulating factor (CSF)-1 is a cytokine that is produced in diabetic kidneys and promotes macrophage accumulation, activation and survival. CSF-1 acts exclusively through the *c-fms* receptor, which is only expressed on cells of the monocyte–macrophage lineage. Therefore, we used *c-fms* blockade as a strategy to selectively target macrophage-mediated injury during the progression of diabetic nephropathy.

Methods Obese, type 2 diabetic *db/db* BL/KS mice with established albuminuria were treated with a neutralising anti-*c-fms* monoclonal antibody (AFS98) or isotype

matched control IgG from 12 to 18 weeks of age and examined for renal injury.

Results Treatment with AFS98 did not affect obesity, hyperglycaemia, circulating monocyte levels or established albuminuria in *db/db* mice. However, AFS98 did prevent glomerular hyperfiltration and suppressed variables of inflammation in the diabetic kidney, including kidney macrophages (accumulation, activation and proliferation), chemokine CC motif ligand 2 levels (mRNA and urine protein), kidney activation of proinflammatory pathways (*c-Jun* amino-terminal kinase and activating transcription factor 2) and *Tnf- α* (also known as *Tnf*) mRNA levels. In addition, AFS98 decreased the tissue damage caused by macrophages including tubular injury (apoptosis and hypertrophy), interstitial damage (cell proliferation and myofibroblast accrual) and renal fibrosis (*Tgf- β 1* [also known as *Tgfb1*] and *Col4a1* mRNA).

Conclusions/interpretation Blockade of *c-fms* can suppress the progression of established diabetic nephropathy in *db/db* mice by targeting macrophage-mediated injury.

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Abbreviations

ATF	Activating transcription factor
CCL2	Chemokine CC motif ligand 2
CCR	Chemokine CC motif receptor
CSF	Colony-stimulating factor
DAB	3,3-Diaminobenzidine
ERK	Extracellular signal-regulated kinase
gcs	Glomerular cross-section
JNK	<i>c-Jun</i> amino-terminal kinase

mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
PLP	Paraformaldehyde–lysine–periodate
SMA	Smooth muscle actin
WT1	Wilm's tumour antigen 1

Introduction

Kidney macrophage accumulation is a feature of diabetic nephropathy that can predict patient decline in renal function [1]. Animal studies suggest that macrophages mediate renal injury in experimental models of diabetic nephropathy [2, 3]. Hyperglycaemia and AGE activate macrophages, resulting in the generation of reactive oxygen species [4] and secretion of proinflammatory cytokines (e.g. TNF- α , IL-1) and pro-fibrotic growth factors (e.g. TGF- β , platelet-derived growth factor) [3, 5, 6]. These macrophage responses exacerbate inflammation, promote tissue injury and stimulate fibrosis, thereby contributing to the development of diabetic nephropathy.

Strategies that reduce macrophage accumulation are reno-protective in inflammatory kidney diseases. For example, macrophage depletion reduces renal injury in rodent models of crescentic glomerulonephritis and renal allograft rejection [7, 8]. Similarly, genetic deficiency of molecules facilitating leucocyte recruitment (e.g. *Icam1*, *Ccl2*) and pharmacological blockade of chemokine CC motif receptors (CCR) 1 and 2 can reduce macrophage accumulation and renal injury in mouse models of diabetic nephropathy [6, 9–12]. However, these approaches are limited in that they do not target macrophages selectively, restricting the interpretation of these findings.

Recent studies show that macrophage accumulation in the kidney and other tissues can be selectively targeted using antibodies or pharmacological compounds that block *c-fms* signalling [13–16]. *c-fms* is a transmembrane receptor, which is present on monocyte–macrophages and osteoclasts and binds colony-stimulating factor (CSF)-1 with high affinity, resulting in receptor autophosphorylation and activation of signalling pathways such as phosphatidylinositol-3 kinase and extracellular signal-regulated kinase (ERK) [17]. CSF-1-induced *c-fms* signalling regulates the proliferation, survival and function of monocytes and fully differentiated macrophages [18–20]. Furthermore, *c-fms*-mediated and integrin-mediated signalling are important in the regulation of macrophage adhesion and motility [21, 22], which facilitate macrophage transmigration. Treatment with a neutralising *c-fms* monoclonal antibody (mAb) suppressed macrophage accumulation in mouse models of renal injury, including unilateral ureteric obstruction and renal allograft rejection

[13, 14]. Similarly, an inhibitor of the *c-fms* receptor kinase prevented kidney macrophage accumulation in a model of ureteric obstruction [15]. These separate strategies both indicate that *c-fms* is a valid therapeutic target for specifically preventing macrophage-mediated renal injury.

Levels of CSF-1 are increased in human and experimental glomerulonephritis and correlate with kidney macrophage proliferation [23]. Studies in glomerulonephritis, ureteric obstruction and diabetic nephropathy have shown that glomerular podocytes and damaged tubules are major sites of CSF-1 production in the injured kidney. Since the development of diabetic nephropathy is thought to be dependent on macrophage-mediated injury [24], we evaluated whether specific targeting of macrophages by *c-fms* blockade would be effective in reducing renal inflammation and injury in established diabetic nephropathy. This was done using a neutralising *c-fms* antibody to treat type 2 diabetic nephropathy in *db/db* mice after the onset of albuminuria.

Methods

Treatment antibodies AFS98 is a rat anti-mouse *c-fms* mAb (IgG2a) which neutralises the activity of *c-fms* by preventing the binding of CSF-1 [13]. AFS98 and an irrelevant control mAb (GL117, IgG2a) were produced from hybridomas and determined to be endotoxin-free before therapeutic use [13].

Animal model Obese (*db/db*) and lean *db/+* heterozygote control mice were created by breeding pairs of C57BL/KS *db/+* mice obtained from Jackson Laboratories (Bar Harbor, ME, USA) and genotyped by PCR for the mutated leptin receptor. Mice were bred in-house at Monash Medical Centre (Clayton, Australia) and maintained on a normal diet under standard animal house conditions. Groups of obese male *db/db* mice ($n=12$) were selected for equivalent hyperglycaemia and albuminuria at 12 weeks of age and were given intraperitoneal injections of anti-*c-fms* mAb (AFS98, 25 mg/kg) or control mAb (GL117, 25 mg/kg) every second day for 6 weeks. Mice were fasted for 3 h every 2 weeks and assessed for body weight, blood glucose by tail vein blood sampling (Medisense glucometer; Abbott Laboratories, Bedford, MA, USA) and urinary albumin excretion. At 18 weeks, mice were killed and serum creatinine, HbA_{1c} and blood monocytes (flow cytometry) were measured and tissues collected and weighed. Additional control groups of 18-week-old non-diabetic *db/+* mice ($n=13$) and 12-week-old untreated diabetic *db/db* mice ($n=8$) were also examined. Tissues were fixed in 4% (vol./vol.) neutral buffered formalin, methyl Carnoy's fixative

(60% methanol, 30% chloroform, 10% glacial acetic acid; vol./vol.) or 2% (wt/vol.) paraformaldehyde–lysine–periodate (PLP), or they were snap-frozen and stored at -80°C . These studies were approved by the Monash Medical Centre Animal Ethics Committee in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 7th edition (2004).

Biochemical analysis Urine was collected from mice housed in metabolism cages for 18 h. Urine albumin and chemokine CC motif ligand 2 (CCL2) levels were measured with ELISA kits (Bethyl Laboratories, Montgomery, TX, USA and BD OptEIA, BD Biosciences, San Diego, CA, USA respectively). Whole mouse blood was collected by cardiac puncture in anaesthetised mice and stored as serum or heparinised plasma. HbA_{1c} and serum and urine creatinine were measured by HPLC [25].

Histopathology analysis Formalin-fixed sections ($2\mu\text{m}$) were stained with periodic acid-Schiff's reagent to assess structure and counterstained with haematoxylin to identify nuclei. Glomerular volume and mesangial matrix fraction were assessed by image analysis (Image Pro Plus; Media Cybernetics, Silver Spring, MD, USA) and cellularity was assessed by counting the total number of nuclei in 20 hilar glomerular cross-sections (gcs) per animal. Tubular atrophy was assessed by counting the number of injured (dilated, atrophied, necrotic) tubular cross sections in ten cortical fields (magnification, $\times 250$) as a percentage of total tubular cross sections. Tubular hypertrophy was assessed by measuring the cross-sectional area of 100 transversely sectioned proximal convoluted tubules (magnification, $\times 400$) per animal, using computer image analysis. All scoring was performed on blinded slides.

Antibodies The primary antibodies used in this study were: (1) rabbit anti-phospho-p38 mitogen-activated protein kinase (MAPK; Thr180/Tyr182), rabbit anti-phospho-p44/42 (Thr202/204), rabbit anti-phospho c-Jun amino-terminal kinase (JNK)1/2 (Thr183/Thy185), rabbit anti-phospho activating transcription factor (ATF)-2 (Thr 69/71) and rabbit anti-cleaved caspase 3 (ASP175; all from Cell Signaling Technology, Beverly, MA, USA); (2) rabbit anti-Wilm's tumour antigen 1 (WT1; Santa Cruz Biotechnology, Santa Cruz, CA, USA); (3) rat anti-mouse Ki-67 (TEC-3; Dako, Carpinteria, CA, USA); (4) mouse anti- α -tubulin and fluorescein-conjugated anti- α -smooth muscle actin (SMA; Sigma, St Louis, MO, USA); (5) goat anti-collagen IV (Southern Biotechnology, Birmingham, AL, USA); and (6) rat anti-CD68 and rat anti-CD169 (Serotec, Oxford, UK). Normal rabbit and goat serum or isotype-matched irrelevant IgGs were used as negative controls.

Immunohistochemistry Immunostaining for α -SMA and collagen IV was performed on $4\mu\text{m}$ sections fixed in methyl Carnoy's solution. Immunostaining for cleaved caspase-3, Ki-67 and WT1 was performed on $4\mu\text{m}$ formalin-fixed paraffin sections. Immunostaining for CD68 and CD169 was performed on $5\mu\text{m}$ PLP-fixed cryostat sections [9]. For retrieval of antigens (except α -SMA, collagen IV, CD68 and CD169) sections were heated in a microwave oven (800 W, 12 min) or pressure cooker (high setting 20 min, full pressure 5 min) in 10 mmol/l sodium citrate buffer (pH6.0). Antigens were then labelled by overnight incubation with primary antibody followed by biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA, USA), and detection was performed using a standard peroxidase-ABC system (Vector) and development with 3,3-diaminobenzidine (DAB; Sigma) [9]. For detection of FITC-conjugated anti- α -SMA mAb, sections were incubated with peroxidase-conjugated sheep anti-fluorescein IgG (Roche Biochemicals, Mannheim, Germany) prior to DAB development [3].

For double labelling of proliferating macrophages, CD68 immunostaining was first performed with DAB development. These sections were then microwaved in citrate buffer and incubated sequentially with Ki-67 mAb, a biotinylated secondary antibody and alkaline phosphatase-conjugated ABC complexes (Vector). They were then developed with nitroblue tetrazolium (NBT) chromogen and bromochloroindolyl phosphate (BCIP) substrate (Roche).

Quantitation of immunohistochemistry Immunostained cells were counted as glomerular⁺ cells/gcs or interstitial⁺ cells/ mm^2 in each animal. CD68^+ , CD169^+ , KI-67^+ cells and WT1 were counted in 25 hilar gcs or 50 cortical fields (magnification, $\times 400$). Cleaved caspase-3⁺ apoptotic cells and proliferating $\text{CD68}^+\text{KI-67}^+$ macrophages were counted in 50 gcs and the entire cortical interstitium. α -SMA immunostaining was assessed by image analysis in the periglomerular area (between Bowman's capsule and surrounding tubules) around 25 hilar gcs (magnification, $\times 400$) and expressed as the percentage of staining around the perimeter of Bowman's capsule. Glomerular collagen IV was assessed as the percentage of area stained in 20 hilar gcs/animal. Tubulo-interstitial collagen IV was assessed by measuring the area stained and counting the number of tubular cross-sections per field (ten fields at $\times 250$ magnification), excluding glomeruli and blood vessels. The ratio of collagen IV/tubules was standardised as the percentage of area stained:100 tubular cross-sections. This method was applied to account for the occurrence of significant tubular hypertrophy with diabetes, leading to a reduction in tubular density (and tubular basement membrane collagen staining) per field.

Western blotting Frozen half kidneys were homogenised and sonicated in lysis buffer as previously described [26]. Lysate proteins were separated on a 4% to 20% (wt/vol.) SDS-PAGE gel and electro-blotted on to nitrocellulose membranes. Membranes were then blocked for 1 h with Odyssey blocking buffer (LI-COR, Lincoln, NB, USA) and incubated overnight with primary antibody in Odyssey buffer at 4°C. Blots were then washed with Tris-buffered saline/0.1% (vol./vol.) Tween-20 and incubated for 1 h with secondary antibody (goat anti-rabbit Alexa Fluor 680 [Invitrogen, Carlsbad, CA, USA] or donkey anti-mouse IRDye 800 [Rockland, Gilbertsville, PA, USA]). After washing, protein bands were detected using an image detection system (Odyssey Infrared; LI-COR). α -Tubulin was used as a loading control. Densitometry analysis was performed using an analyser (Gel-Pro Analyzer 3.0; Media Cybernetics). Results were expressed as the integrated optical density relative to tubulin.

Real-time PCR Total RNA was extracted from whole kidney samples using RiboPure reagent (Ambion, Austin, TX, USA) and reverse-transcribed using a kit (Superscript First-Strand Synthesis kit; Invitrogen) with random primers. Real-time PCR was performed on Rotor-Gene 3000 (Corbett Research, Sydney, NSW, Australia) with thermal cycling conditions of 37°C for 10 min and 95°C for 5 min, followed by 50 cycles of 95°C for 15 s, 60°C for 20 s and 68°C for 20 s. The primer pairs and carboxyfluorescein-labelled minor groove binder probes used are indicated in the Electronic supplementary material (ESM) Table 1. The relative amount of mRNA was calculated using the comparative C_t ($\Delta\Delta C_t$) method. All specific amplicons were normalised against 18S rRNA, which was amplified in the same reaction as an internal control using commercial assay reagents (Applied Biosystems, Foster City, CA, USA).

Statistical analysis Statistical differences between two groups were analysed by the unpaired Student's *t* test. Differences between multiple groups were analysed by one way ANOVA with Tukey's multiple comparison post test. Correlations were performed using Pearson's correlation coefficient. Data were recorded as mean $\pm$ SEM and $p < 0.05$ was considered significant. All analyses were performed using GraphPad Prism 5.0 (GraphPad, San Diego, CA, USA).

Results

Kidney levels of CSF-1 are increased in *db/db* mice during early diabetic renal injury In contrast to non-diabetic *db/+*

controls, diabetic *db/db* mice showed signs of renal injury as early as 12 weeks of age, indicated by an elevated rate of urine albumin excretion (*db/db* $252 \pm 44 \mu\text{g}/24 \text{ h}$ vs *db/+* $18 \pm 4 \mu\text{g}/24 \text{ h}$, $p < 0.0001$). This development of renal injury in *db/db* mice was associated with a 36-fold increase in the relative level of kidney expression of *Csf1* mRNA:18S rRNA (*db/db* 36.1 ± 5.6 vs *db/+* 1.0 ± 0.2 , $p < 0.0001$) and a rise in CD68⁺ glomerular macrophages (*db/db* 1.35 ± 0.10 vs *db/+* 0.60 ± 0.13 cells/gcs, $p < 0.0001$), suggesting that kidney production of CSF-1 may contribute to the onset and progression of diabetic nephropathy by promoting renal inflammation.

***c-fms* antibody treatment reduces renal hypertrophy, but not obesity, diabetes or circulating monocytes in *db/db* mice** Diabetic *db/db* mice experienced mild weight loss between weeks 12 and 18 due to worsening hyperglycaemia, which was equivalent in mice treated with control or anti-*c-fms* (AFS98) mAb (Fig. 1). HbA_{1c} levels in *db/db* mice at 18 weeks also demonstrated that AFS98 had no impact on hyperglycaemia during the treatment period (Fig. 1). Similarly, the peripheral monocyte count was equivalent in *db/db* mice receiving AFS98 or control mAb (65 ± 11 vs 64 ± 17 cells/ μl , respectively, $p = 0.9$) at 18 weeks, suggesting that AFS98 treatment does not deplete circulating monocytes. In addition, diabetic *db/db* mice developed significant hepatomegaly, visceral adiposity and renal hypertrophy, but only renal hypertrophy was reduced with AFS98 treatment (Table 1).

***c-fms* antibody reduces macrophage accumulation in diabetic kidneys** Control diabetic *db/db* mice (receiving control mAb) showed a 2.4-fold increase in immunostaining for total (CD68⁺) glomerular macrophages and a tenfold increase in activated (CD169⁺) glomerular macrophages at 18 weeks of age, compared with non-diabetic *db/+* controls (Table 2). AFS98 treatment reduced the total number of glomerular macrophages by 60% in *db/db* mice, but the number of glomerular CD169⁺ macrophages remained similar to control *db/db* mice (Table 2). The effect of AFS98 was more pronounced on kidney interstitial macrophages. The number of CD68⁺ and CD169⁺ interstitial macrophages were increased by 20% and 86%, respectively, at 18 weeks of age in diabetic *db/db* mice compared with *db/+* mice (Table 2). AFS98 treatment not only prevented this increase, but also reduced CD68⁺ and CD169⁺ interstitial macrophages to about 30% of normal levels (Table 2, Fig. 2a, b). Double immunostaining for CD68 and Ki-67 demonstrated that glomerular and interstitial macrophage proliferation (CD68⁺Ki-67⁺ cells) was increased in control diabetic *db/db* mice compared with normal *db/+* mice (Fig. 2c–f). Kidney macrophage proliferation in diabetic *db/db* mice was significantly

Fig. 1 Obesity and hyperglycaemia are unaffected by AFS98 treatment. The body weight profiles (**a**) show that diabetic *db/db* mice treated with control mAb (triangles) and AFS98 mAb (inverted triangles) maintained a similar degree of obesity during the study compared with non-diabetic *db/+* controls (circles). **b** The fasting blood glucose levels showed that both groups of *db/db* mice

developed a similar hyperglycaemia profile, whereas *db/+* mice maintained normal blood glucose levels. **c** HbA_{1c} measured at 18 weeks demonstrated that hyperglycaemia had been equivalent in both groups of diabetic *db/db* mice during the study. Data are means $\pm$ SEM; $n=12$ –13. *** $p<0.001$

reduced with AFS98 treatment (Fig. 2e, f); however, only about 5% of glomerular and 1% of interstitial macrophages were found to be proliferating in diabetic *db/db* kidneys.

c-fms antibody suppresses diabetic glomerular hyperfiltration without altering albuminuria At 18 weeks of age, control diabetic *db/db* mice showed a threefold increase in creatinine clearance compared with *db/+* mice, indicating significant glomerular hyperfiltration. Treatment with AFS98 prevented this hyperfiltration (Fig. 3a). Albuminuria was well established in diabetic *db/db* mice at the initiation of treatment (week 12; Fig. 3b), which coincided with loss of glomerular podocytes (Fig. 3c). Albuminuria continued to rise during treatment without further reduction in podocytes; however, AFS98 treatment did not alter the development of albuminuria or podocyte number (Fig. 3b, c).

c-fms antibody reduces tubular injury in diabetic *db/db* mice Histological morphometric analysis of diabetic *db/db* glomeruli identified hypertrophy, hypercellularity and an increase in the mesangial matrix fraction, which were unaffected by AFS98 treatment (Table 3). However, immunostaining indicated that the glomerular deposition

of collagen IV was partially reduced with AFS98 treatment (Table 3). Compared with *db/+* mice, the kidneys of control diabetic *db/db* mice showed tubular hypertrophy with increased tubular apoptosis and proliferation. AFS98 treatment reduced tubular hypertrophy in *db/db* mice, which appeared to account for the reduction in overall kidney size (Fig. 4, Table 3). AFS98 treatment also normalised tubular apoptosis and reduced tubular proliferation in diabetic kidneys (Table 3). At this early stage of diabetic nephropathy, tubular atrophy was not prominent and was indistinguishable between the *db/db* groups. Consequently, other markers of tubular injury, namely urinary CCL2 and kidney *Kim1* (also known as *Havr1*) mRNA levels, were evaluated. The control diabetic *db/db* mice showed a twofold increase in urinary CCL2 and a threefold increase in whole kidney *Kim1* mRNA, compared with *db/+* mice. Both these markers of tubular injury were normalised with AFS98 treatment (Fig. 4). Interstitial macrophage accumulation showed a significant correlation with markers of tubular injury, including tubular hypertrophy ($r=0.56$, $p=0.0079$), tubular apoptosis ($r=0.51$, $p=0.016$), urinary CCL2 ($r=0.73$, $p=0.0001$) and kidney *Kim1* mRNA ($r=0.60$, $p=0.0007$).

Table 1 Characteristics of experimental mice at 18 weeks

Strain:	<i>db/+</i>	<i>db/db</i>	
Treatment:	Nil	Control mAb	AFS98 mAb
Body weight (g)	29.2 $\pm$ 2.1	41.2 $\pm$ 7.7 ^a	42.7 $\pm$ 7.6 ^a
Epididymal fat (g)	0.54 $\pm$ 0.04	2.19 $\pm$ 0.15 ^a	2.08 $\pm$ 0.19 ^a
Liver (g)	1.23 $\pm$ 0.03	2.53 $\pm$ 0.23 ^a	2.51 $\pm$ 0.11 ^a
Total kidney weight (g)	0.35 $\pm$ 0.01	0.50 $\pm$ 0.02 ^a	0.44 $\pm$ 0.01 ^{a,b}

Data are mean $\pm$ SEM, $n=12$ –13

^a $p<0.001$ vs *db/+* mice

^b $p<0.05$ vs control mAb-treated *db/db* mice

Table 2 Kidney macrophage accumulation and activation

Strain:	<i>db/+</i>	<i>db/db</i>	
Treatment:	Nil	Control mAb	AFS98 mAb
Glomerular macrophages			
CD68 ⁺ (cells/gcs)	0.67±0.07	1.60±0.20 ^a	1.03±0.23 ^d
CD169 ⁺ (cells/gcs)	0.03±0.01	0.37±0.07 ^b	0.40±0.11 ^b
Interstitial macrophages			
CD68 ⁺ (cells/mm ²)	81.0±4.0	95.0±4.0 ^a	21.9±2.4 ^{c,e}
CD169 ⁺ (cells/mm ²)	17.4±2.1	32.3±3.1 ^c	4.9±0.6 ^{b,c}

Data are mean ± SEM, *n*=12–13^a*p*<0.05, ^b*p*<0.01, ^c*p*<0.001 vs *db/+* mice^d*p*<0.05, ^e*p*<0.001 vs control mAb-treated *db/db* mice

c-fms antibody reduces renal fibrosis in diabetic *db/db* mice Control diabetic *db/db* kidneys displayed greater numbers of apoptotic and proliferating interstitial cells and an increased accumulation of periglomerular α -SMA⁺

Fig. 2 AFS98 treatment reduces macrophages in diabetic kidneys. Immunostaining identified many interstitial CD68⁺ macrophages (brown) in the kidneys of diabetic *db/db* mice treated with **a** control mAb; this was markedly reduced by treatment with **b** AFS98 mAb. Magnification, $\times 400$. **c, d** Proliferating kidney macrophages (arrows) were detected by dual immunostaining for CD68 (brown) and KI-67 (blue) in a **c** glomerulus and the **d** interstitium of diabetic *db/db* mice treated with control mAb. Magnification, $\times 1,000$. At 18 weeks of age, AFS98 treatment had reduced the number of proliferating macrophages in **e** glomeruli and the **f** interstitium of diabetic *db/db* mice compared with those receiving control mAb. Data are means $\pm$ SEM; *n*=12–13. **p*<0.05, ***p*<0.01, ****p*<0.001

myofibroblasts and tubulo-interstitial deposition of collagen IV, compared with *db/+* kidneys. AFS98 treatment reduced the number of proliferating interstitial cells, the accumulation of periglomerular myofibroblasts and the tubulo-interstitial deposition of collagen IV in diabetic *db/db* mice (Table 3, Fig. 5). In support of these findings, we found that kidney mRNA levels of *Col4a1* and *Tgf- β 1* (also known as *Tgfb1*) were increased approximately threefold in control diabetic *db/db* compared with *db/+* mice, but were normalised by AFS98 treatment (Fig. 5). Interstitial macrophage accumulation correlated significantly with the number of proliferating interstitial cells ($r=0.81$, $p<0.0001$), periglomerular α -SMA staining ($r=0.48$, $p=0.0257$), tubulo-interstitial collagen IV staining ($r=0.66$, $p=0.0022$) and kidney *Tgf- β 1* mRNA levels ($r=0.50$, $p=0.0195$).

c-fms antibody attenuates activation of pro-inflammatory mechanisms Compared with *db/+* mice, control diabetic *db/db* mice showed a 2.3-, 1.5- and 3.8-fold increase in kidney levels of phospho-ERK, phospho-p38 MAPK and phospho-JNK by western blotting, respectively (Fig. 6). AFS98 treatment of *db/db* mice did not alter the kidney levels of phospho-ERK or phospho-p38 MAPK, but did reduce the levels of phospho-JNK and phosphorylation of the downstream transcription factor, ATF2. In addition, kidney mRNA levels of *Tnf- α* and *Ccl2* were increased by threefold in control diabetic *db/db* compared with *db/+* mice, this increase being prevented by AFS98 treatment (Fig. 7). Kidney interstitial macrophage accumulation correlated with kidney levels of phospho-ATF2 ($r=0.56$, $p=0.0066$) and the mRNA levels of *Tnf- α* ($r=0.71$, $p=0.0002$) and *Ccl2* ($r=0.50$, $p=0.0203$).

Discussion

This study demonstrates that CSF-1 plays a significant role in the development of type 2 diabetic nephropathy through its interaction with macrophage *c-fms*.

AFS98 treatment did not affect the obesity or progression of diabetes in *db/db* mice. Previous studies indicate that macrophage infiltration into adipose tissue promotes

Fig. 3 AFS98 treatment reduces glomerular hyperfiltration but not albuminuria. **a** Assessment of creatinine clearance at 18 weeks of age identified glomerular hyperfiltration in *db/db* mice treated with control mAb compared with non-diabetic *db/+* controls; this was prevented by treatment with AFS98. **b** Compared with non-diabetic *db/+* mice (circles), the urine albumin excretion rate was markedly elevated at 12 to 18 weeks in *db/db* mice receiving control mAb (triangles) and was

unaffected by AFS98 treatment (inverted triangles). **c** Podocyte numbers were significantly reduced in diabetic mice (black bars) at week 12 compared with non-diabetic controls (white bars) and were similarly diminished at week 18 of diabetes in mice treated with AFS98 or control mAb (G117). Data are means $\pm$ SEM; $n=12$ –13. *** $p<0.001$

insulin resistance in insulin-sensitive tissues, leading to type 2 diabetes [27–29]. Consequently, *c-fms* antibody blockade could have reduced the accumulation of adipose macrophages, thereby restoring insulin sensitivity and lowering hyperglycaemia. However, since the fasting blood glucose profile and HbA_{1c} levels were unaffected by AFS98 treatment, we did not explore whether this treatment altered adipose macrophages in our *db/db* mice.

Our primary outcome was that AFS98 treatment reduced kidney macrophages in diabetic *db/db* mice. Both direct and indirect mechanisms facilitating macrophage accumulation

were affected. As expected, AFS98 suppressed local macrophage proliferation in diabetic kidneys; however, given that only 1% to 5% of total kidney macrophages were proliferating at week 18, it is likely that our *c-fms* blocking strategy contributed to the reduction in kidney macrophages by additional mechanisms. AFS98 did not cause any significant difference in apoptosis of interstitial cells at week 18; however, it did reduce the interstitial macrophage numbers to 30% of normal levels, suggesting that this treatment may decrease the duration of macrophage survival. AFS98 treatment may also have indirectly

Table 3 Renal pathology

Strain:	<i>db/+</i>	<i>db/db</i>	
Treatment:	Nil	Control mAb	AFS98 mAb
Glomerular injury			
Volume ($\mu\text{m}^3 \times 10^4$)	15.6 $\pm$ 0.8	25.1 $\pm$ 0.8 ^a	26.4 $\pm$ 0.8 ^a
Cellularity (cells/gcs)	29.8 $\pm$ 0.4	34.4 $\pm$ 0.7 ^a	33.3 $\pm$ 0.6 ^a
Matrix fraction (%)	23.1 $\pm$ 0.5	32.7 $\pm$ 0.9 ^a	32.7 $\pm$ 0.5 ^a
Collagen IV (%)	14.1 $\pm$ 0.6	25.0 $\pm$ 0.7 ^c	22.3 $\pm$ 0.5 ^{c,d}
Tubular injury			
Proximal tubule CSA (μm^2)	948 $\pm$ 10	1,112 $\pm$ 24 ^d	1,050 $\pm$ 13 ^{b,c}
Atrophic (%)	0.10 $\pm$ 0.08	0.55 $\pm$ 0.35	0.34 $\pm$ 0.20
Apoptosis (act-CSP3+ cells/mm ²)	0.20 $\pm$ 0.03	0.32 $\pm$ 0.04 ^a	0.17 $\pm$ 0.03 ^c
Proliferation (%)	8.5 $\pm$ 0.8	13.8 $\pm$ 1.7 ^a	10.4 $\pm$ 1.4
Interstitial injury			
Periglomerular α -SMA (%)	8 $\pm$ 1	35 $\pm$ 3 ^c	25 $\pm$ 2 ^{c,d}
Collagen IV (%)	10.8 $\pm$ 0.2	13.2 $\pm$ 0.5 ^c	11.2 $\pm$ 0.3 ^{c,d}
Apoptosis (act-CSP3+ cells/mm ²)	0.19 $\pm$ 0.06	0.37 $\pm$ 0.06	0.30 $\pm$ 0.05
Proliferation (cells/mm ²)	5.3 $\pm$ 0.5	9.8 $\pm$ 1.4 ^b	3.5 $\pm$ 0.6 ^f

Data are mean $\pm$ SEM, $n=12$ –13

^a $p<0.05$, ^b $p<0.01$, ^c $p<0.001$ vs *db/+* mice

^d $p<0.05$, ^e $p<0.01$, ^f $p<0.001$ vs control mAb-treated *db/db* mice
act-CSP3, activated caspase-3; CSA, cross-sectional area

Fig. 4 AFS98 treatment reduces tubular damage. **a** Morphometric analysis of the transverse cross-sectional area of proximal convoluted tubules showed that diabetic *db/db* mice receiving control mAb had significant hypertrophy compared with normal *db/+* mice at 18 weeks of age, which was reduced by AFS98 treatment. Tubular injury in *db/db* mice treated with control mAb was identified by increases in **b** tubular apoptosis, **c** urine excretion of CCL2:creatinine and **d** kidney levels of *Kim1* vs 18S RNA compared with *db/+* mice at 18 weeks of age. Injury was prevented by AFS98 treatment. Data are means $\pm$ SEM; $n=12-13$. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

suppressed macrophage recruitment by reducing production of CCL2 in diabetic kidneys. In addition, *c-fms* blockade may inhibit monocyte–macrophage migration through regulation of macrophage adhesion and motility [21, 22] and by preventing kidney macrophages from producing their own monocyte/macrophage chemoattractants, including CSF-1. Indeed, CSF-1 can be a potent chemoattractant for cells expressing high levels of *c-fms* [30] and may also serve to recruit monocytes from the circulation [31].

It was surprising that AFS98 reduced the number of activated (CD169⁺) macrophages in the interstitium, but not in the glomeruli of diabetic *db/db* mice. One explanation is that circulating immune complexes, capable of activating macrophages, accumulate in the glomeruli, but not in the interstitium of diabetic *db/db* kidneys [2]. In this study, we detected glomerular deposition of IgG and C3 by immunofluorescence in *db/db* mice, with no difference between control and treated groups (data not shown). Renal biopsy studies have also identified glomerular deposits of immune complexes in diabetic patients [32]. Another possibility is

Fig. 5 AFS98 treatment reduces tubulo-interstitial fibrosis. Immunostaining for α -SMA (brown) (**a–c**) showed that its production was confined to blood vessels in **a** non-diabetic *db/+* kidney. Periglomerular and interstitial levels of α -SMA were seen in accumulating myofibroblasts in a diabetic kidney from **b** a *db/db* mouse receiving control mAb; levels were reduced in **c** a diabetic *db/db* mouse treated with AFS98 mAb. Magnification, $\times 400$. **d–f** Immunostaining identified collagen IV deposition (brown) in the tubular and glomerular basement membranes and the mesangium of **d** non-diabetic *db/+* mice. Additional collagen IV deposition was found in the expanded mesangium and interstitial areas of a diabetic kidney from **e** a diabetic *db/db* mouse given control mAb; deposition was reduced in **f** a diabetic *db/db* mouse treated with AFS98 mAb. Magnification, $\times 250$. **g** Quantitation of the kidney area staining for α -SMA and collagen IV (**h**) verified that AFS98 reduces interstitial myofibroblasts and collagen IV accumulation. **i** Diabetic *db/db* mice receiving control mAb had increased kidney levels of *Tgf- β* mRNA and **j** *Col4a1* compared with non-diabetic *db/+* mice; increases were prevented by AFS98 treatment. Data are means $\pm$ SEM; $n=12-13$. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

Fig. 6 Effects of AFS98 treatment on MAPK signalling in diabetic kidneys. **a** Representative western blots show the kidney levels of phospho-ERK, phospho-p38, phospho-JNK, phospho-ATF2 and α-tubulin (loading control) in *db/+* mice and diabetic *db/db* mice receiving control mAb and AFS98. **b–e** Densitometry analysis demonstrated that kidney levels of each of the above proteins were increased in untreated diabetic *db/db* mice compared with *db/+* mice. The elevated kidney levels of **b** phospho-ERK and **c** phospho-p38 in diabetic *db/db* mice given control mAb were not different from those of mice given AFS98 treatment. However, the elevated kidney levels of **d** phospho-JNK and **e** phospho-ATF2 in *db/db* mice receiving control mAb were reduced by AFS98 treatment. Data are means ± SEM; *n*=12–13. **p*<0.05, ***p*<0.01, ****p*<0.001

that the activation of glomerular macrophages is regulated indirectly by the effects of the diabetic milieu on mesangial cells or podocytes through cytokine secretion or cell–cell interactions and that these are unaffected by AFS98. Indeed

Fig. 7 AFS98 treatment suppresses gene transcription of proinflammatory cytokines. Diabetic *db/db* mice receiving control mAb had a threefold increase in kidney mRNA levels of **a** *Tnf-α* and **b** *Ccl2* compared with *db/+* mice; increases were prevented by AFS98 treatment. Data are means ± SEM; *n*=12–13. ***p*<0.01, ****p*<0.001

the differing microenvironments of the glomerulus and the tubulo-interstitium can influence the macrophage phenotype [9], which may be an important feature of diabetic nephropathy.

Our second main finding was that renal hyperfiltration was prevented by AFS98 treatment, suggesting a role for macrophages in this process. AFS98 reduced hypertrophy of the kidney and proximal tubules, which may have resulted in reduced tubular re-absorption. In diabetic kidneys, tubular re-absorption is usually increased, a finding thought to be the dominant cause of hyperfiltration operating through a tubulo-glomerular feedback mechanism [33]. Therefore, macrophages may stimulate diabetic hyperfiltration by promoting processes that increase tubular re-absorption. In addition, tubules are known to secrete creatinine [34], which also may be increased by macrophage-mediated injury during diabetes, contributing to the apparent increase in creatinine clearance.

Although treatment with AFS98 reduced renal inflammation and hyperfiltration, it had no effect on levels of established albuminuria in *db/db* mice. Several factors may account for this. Our analysis showed that irreversible podocyte loss had occurred in diabetic glomeruli during the establishment of albuminuria prior to treatment. *c-fms* blockade was unable to repair this damage. AFS98 treatment was also unable to reduce the number of activated glomerular macrophages, which may be critical for promoting albuminuria. In addition, albuminuria before treatment may have reached a level that caused sufficient ongoing injury to the kidney to be self-sustaining and therefore not reversible by anti-*c-fms* therapy. These concepts are supported by a previous study, which similarly showed that intervention treatment with a CCR1 antagonist was able to reduce interstitial inflammation, but not albuminuria in an accelerated model of type 2 diabetes

[11]. This study and ours both suggest that interstitial macrophages are not important for the maintenance of overt albuminuria in diabetic kidney disease. It is also noteworthy that the protection from hyperfiltration achieved with AFS98 did not affect the albuminuria level in these diabetic mice. One possible interpretation is that AFS98 treatment also induced an increase in glomerular permeability to albumin. However, this finding could also be explained by AFS98 reducing tubular re-absorption of filtered albumin.

Our third important finding was that AFS98 treatment protected diabetic *db/db* kidneys from tubulo-interstitial damage, which included a reduction in tubular injury (hypertrophy, proliferation, apoptosis and mRNA levels of *Kim1*) and interstitial fibrosis (interstitial myofibroblast accumulation and tubulo-interstitial deposition of collagen IV). These results provide convincing evidence that macrophages promote tubular injury and interstitial fibrosis in diabetic kidneys, a concept proposed by previous studies [3,11]. In the current analysis, we noted that *c-fms* blockade was associated with a reduction in kidney mRNA levels of inflammatory cytokines (*Tnf- α* , *Ccl2*), a profibrotic cytokine (*Tgf- β 1*) and *Col4a1*. Production of these mediators is known to be regulated by intracellular MAPK signalling, which is induced by elements of the diabetic milieu. Our examination of MAPK activation in the diabetic kidneys found that AFS98 treatment caused significant attenuation of JNK signalling and reduced activation of the downstream transcription factor ATF2. These results suggest that the effects of *c-fms* blockade are at least partly mediated by a reduction in JNK activity, which presumably regulates the functions of macrophages and other renal cells. Other studies have indicated that the activation of JNK following *c-fms* signalling is critical for the development, proliferation and survival of macrophages [35]. In addition, JNK signalling stimulates macrophage production of proinflammatory mediators such as TNF- α and IL-1, which can cause injury to other renal cells or further exacerbate the inflammatory response [36]. Indeed, recent evidence in non-diabetic kidney disease suggests that the activation of macrophages and their potential to cause renal injury are partly dependent on JNK signalling [37]. However, it is also possible that the reduction of kidney JNK signalling in AFS98-treated mice is due to the depletion of macrophages and their ability to induce JNK signalling in other renal cells. Other studies have shown that JNK signalling can promote apoptosis of tubular cells [38, 39], the formation of myofibroblasts [40, 41] and TGF- β -induced matrix production [42]. Therefore, JNK signalling may be an important therapeutic target for inhibiting macrophage-mediated injury in diabetic nephropathy.

In conclusion, this study shows that specific targeting of macrophages with a neutralising *c-fms* antibody can suppress the development of inflammation, tissue injury and fibrosis

in the kidneys of a mouse model of type 2 diabetes with established albuminuria. This evidence supports the concept that macrophages play a critical role in the development of diabetic nephropathy. In addition, our findings indicate that the development of inhibitors of *c-fms* or JNK signalling may provide a strategy for protecting diabetic kidneys against macrophage-mediated injury.

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