Endothelial dysfunction and inflammation in diabetic nephropathy

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Chapter 6

Soluble fms-like tyrosine kinase-1 reduces inflammation in *APOC1* transgenic mice

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Abstract

A polymorphism in the *APOC1* gene, which encodes apolipoprotein C-I (apoCI), has been associated with the development of diabetic nephropathy. In addition, patients with diabetes have increased plasma levels of apoCI compared to non-diabetic control subjects. *APOC1* transgenic (*APOC1*-tg) mice develop nodular glomerulosclerosis by the age of 15 months; however, the slow disease progression in this model makes it poorly suited for studying therapeutic and preventive interventions. In an attempt to accelerate the progression of glomerulosclerosis in *APOC1*-tg mice, we introduced a second genetic 'hit' by transfecting these mice with the *sFlt-1* gene, which encodes soluble fms-like tyrosine kinase-1 (sFLT-1), an inhibitor of vascular endothelial growth factor A (VEGF-A). We then investigated whether inducing a systemic increase in sFLT-1 levels for 15 weeks in 8-week-old *APOC1*-tg mice accelerates the development of glomerulosclerosis. Our results demonstrate that a systemic increase in sFLT-1 levels for 15 weeks does not induce the development of glomerulosclerosis in *APOC1*-tg mice. Instead, *APOC1*-tg mice transfected with *sFlt-1* had significantly fewer glomerular macrophages compared to non-transfected *APOC1*-tg mice ($p<0.01$). Surprisingly, the atopic dermatitis that normally develops in *APOC1*-tg mice was resolved following transfection with *sFlt-1*, presumably by reducing the infiltration of inflammatory cells into the skin. Moreover, we found no significant change in glomerular endothelial cell activation or glomerular *VEGF-A* mRNA levels in *sFlt-1*–transfected *APOC1*-tg mice compared to non-transfected *APOC1*-tg mice. Taken together, these data suggest that sFLT-1 has immune-modulating properties independent of its inhibitory effects on VEGF-A.

Introduction

Diabetic nephropathy remains the leading cause of end-stage renal disease worldwide[1]. Although the risks of acute myocardial infarction, stroke, and amputation among diabetic patients have decreased considerably over the past two decades, the risk of developing end-stage renal disease has remained high in these patients[2]. Thus, new preventive and therapeutic strategies, as well as a clearer understanding of the mechanisms that underlie the development and repair process in diabetic nephropathy, are urgently needed.

The risk factors associated with the development of diabetic nephropathy include hyperglycaemia, hyperlipidaemia, hypertension, and genetic factors. With respect to genetics, a polymorphism in the *APOC1* gene, which encodes apolipoprotein C-I (apoCI), has been associated with an increased risk of developing diabetic nephropathy[3]. Furthermore, compared to non-diabetic control subjects, patients with diabetes have increased plasma levels of apoCI[4]. Recently, we reported that patients with diabetic nephropathy have higher levels of glomerular apoCI protein compared to both diabetic patients without nephropathy and non-diabetic controls[5]. We also reported that transgenic mice carrying the *APOC1* transgene (*APOC1*-tg mice), but not wild-type mice, develop albuminuria and nodular glomerulosclerosis, features that are highly reminiscent of diabetic nephropathy. In addition, apoCI increases the inflammatory response of activated macrophages *in vitro*; specifically, these cells have increased expression of tumour necrosis factor alpha (TNF-alpha)[5,6]. Moreover, TNF-alpha production by glomerular macrophages has been suggested to promote the development of diabetic nephropathy[7]. Together, these data support the hypothesis that apoCI increases the inflammatory response of glomerular macrophages in *APOC1*-tg mice, resulting in the development of nodular glomerulosclerosis. Unfortunately, however, *APOC1*-tg mice do not develop glomerulosclerosis until 15 months of age[5]; thus, the relatively slow disease progression in this model makes it unsuitable for studying therapeutic and preventive interventions.

Vascular endothelial growth factor A (VEGF-A) is another important mediator of kidney disease, and studies have shown that VEGF-A plays an important role in the maintenance and survival of endothe-

lial cells[8]. A decrease in glomerular VEGF-A levels below physiological levels results in the development of renal disease. Specifically, mice that lack VEGF-A expression in the glomeruli are not viable and have a wide range of endothelial cell defects; moreover, mice that are heterozygous for VEGF-A selectively in podocytes (i.e. mice with podocyte-specific VEGF-A haploinsufficiency) develop proteinuria, endotheliosis, and progressive loss of endothelial cells[9]. Similarly, treating rats with soluble fms-like tyrosine kinase-1 (sFLT-1) – an inhibitor of VEGF-A – causes endothelial cell damage and loss, hypertension, and proteinuria[10]. Together, these findings indicate that too little VEGF-A and too much sFLT-1 can both lead to glomerular endothelial dysfunction.

In an attempt to accelerate the progression of glomerulosclerosis in *APOC1*-tg mice, we introduced a second genetic 'hit' by transfecting 8-week-old mice with *sFlt-1*. We then examined whether systemically increasing sFLT-1 levels leads to an accelerated development of glomerulosclerosis by monitoring the mice for 15 weeks post-transfection.

Methods

sFlt-1 transfection

Two pcDNA3.1 vectors (Invitrogen, Breda, the Netherlands) containing either *sFlt-1-VSV* or the luciferase gene were generated as described previously[11,12]. The resulting plasmids were amplified in DH5α *E. coli* (Invitrogen), purified using the QIAfilter Plasmid Maxi-prep kit (Qiagen, Venlo, the Netherlands), and dissolved in Endo-Free Tris–EDTA buffer (Qiagen). Mice were transfected by electroporation of the *sFlt-1-VSV* and luciferase constructs into both gastrocnemius muscles (20 μg each) as described previously[11]. To monitor transfection efficiency, the mice were injected intraperitoneally with luciferin at 2-week intervals. Five minutes after each luciferin injection, luciferase activity was visualised at the transfection sites using a Night-OWL bioluminescence camera (Xenogen Ivis Spectrum, Alameda, CA) as described previously[11].

Animals

For this study, we used 8-week-old female C57BL/6J mice (Harlan Laboratories, Indianapolis, IN) and *APOC1*-tg mice. All experiments were conducted in accordance with national guidelines for the care and use of experimental animals (DEC licence 13163). Mice were housed in individually ventilated cages in groups of up to five mice/cage, with food and water provided *ad libitum*. For the experiments, the mice were randomly assigned to groups. At 8 weeks of age, the mice were transfected with *sFlt-1*. The development of glomerulosclerosis was then monitored in *sFlt-1*–transfected and non-transfected *APOC1*-tg mice (n=6 mice/group), as well as in *sFlt-1*–transfected and non-transfected C57BL/6J (wild-type) mice (n=6 mice/group). Fifteen weeks after transfection, the mice were sacrificed; this time point was chosen because we previously found that treatment with sFLT-1 for 15 weeks results in significant changes in kidney function and morphology[12]. One mouse in the non-transfected *APOC1*-tg group was removed from the study due to the development of severe atopic dermatitis complicated by open wounds.

Measurement of the urine albumin:creatinine ratio

To measure the urine albumin:creatinine ratio, spot urine was collected at 5 and 15 weeks after the start of the experiment. Urine albumin levels were measured using rocket immunoelectrophoresis with a rabbit anti-mouse albumin; purified mouse serum albumin (Sigma-Aldrich, Saint Louis, MO) was used as a standard. Urine creatinine was measured using a creatinine assay, with picric acid, sodium hydroxide, and creatinine standards (Sigma-Aldrich); the albumin:creatinine ratio was then calculated.

Immunohistochemistry

Paraffin-embedded kidney tissues were cut at 4-µm thickness using a Leica microtome and stained with periodic acid-Schiff (PAS) using a standard protocol. A rabbit anti-human Wilms tumour 1 (WT1; 1:500; Santa Cruz Biotechnology, Dallas, TX) primary antibody was used for immunostaining, followed by an anti-rabbit-Envision HRP-conjugated

secondary antibody (Dako, Glostrup, Denmark), with diaminobenzidine (DAB+; Dako) as the chromogen. This rabbit anti-human WT1 antibody cross-reacts with mouse WT1 (data not shown). A non-specific isotype-matched antibody was used as a negative control.

Paraffin-embedded skin tissues were cut at 4-µm thickness using a Leica microtome and stained with hematoxylin and eosin (H&E) using a standard protocol. The rat anti-mouse F4/80 (1:100; kindly provided by the Department of Human Genetics, LUMC) primary antibody was used for immunostaining, followed by the anti-rat-Impress (Vector Laboratories, Burlingame, CA) HRP-conjugated secondary antibody, with DAB+ as the chromogen.

Frozen kidney tissues were cut at 4-µm thickness using a Reichert cryostat and immunostained using the following primary antibodies: rat anti-mouse CD68 (1:15; Abcam, Cambridge, MA), rat anti-mouse vascular cell adhesion molecule-1 (VCAM-1; 1:1400; BD Pharmingen, San Diego, CA), rat anti-mouse intercellular adhesion molecule-1 (ICAM-1; 1:200; ATCC, Manassas, VA), and rabbit anti-vesicular stomatitis virus (VSV; 1:2500; Sigma-Aldrich), followed by the appropriate Envision (Dako) or Impress (Vector Laboratories) HRP-conjugated secondary antibody, with DAB+ as the chromogen. For each immunostaining experiment, the respective non-specific isotype-matched antibody was used as a negative control.

Frozen skin tissues were cut at 4-µm thickness using a Reichert cryostat and immunostained with rat anti-mouse CD3 (1:20; Abcam), followed by the appropriate Impress (Vector Laboratories) HRP-conjugated secondary antibody, with DAB+ as the chromogen.

Digital image analysis

Images of tissue sections were digitised using a Philips Ultra-Fast Scanner 1.6 RA. The surface area of the glomerular tuft was measured in PAS-stained slides containing 25 glomeruli per section using Philips Ultra-Fast Scanner 1.6 RA software. ImageJ (NIH, Bethesda, MD) was used to quantify the levels of VCAM-1 and ICAM-1. The positive area per glomerulus was determined by measuring the positively stained area (corrected for the total area of the glomerulus) at 400X magnification with 10 glomeruli per section. The number of podocytes in each sample was determined by counting the number of WT1-positive

nuclei per glomerulus, measured in 25 glomeruli. The number of macrophages was determined by counting the number of CD68-positive cells in 10 glomeruli per section. The glomeruli that were used for these measurements were selected at random. The average thickness of the epidermal layer was determined by averaging 10 measurements per field in 5 fields at 20X magnification using Philips Ultra-Fast Scanner 1.6 RA software.

RNA isolation and quantitative real-time PCR

Frozen kidney tissues were chopped and then dissolved in 1 ml TRIzol (Ambion, Foster City, CA) to obtain total RNA. Quantitative real-time PCR (qRT-PCR) was then performed using SYBR Green I master mix (Bio-Rad, Hercules, CA) in accordance with the manufacturer's instructions; the reactions were run on a Bio-Rad CFX real-time system. Ct (cycle threshold) values were normalised to the housekeeping gene *Hprt1*. The following primers were used: *Hprt1*: 5'-GGCTATAAGTTCTTTGCTGACCTG-3' and 5'-AACTTTTATGTCCCCCGTTGA-3'; *Vegf-a*: 5'-CTGGACCCTGGCTTTACTGC-3' and 5'-GCTTCGCTGGTAGACATCCA-3'.

Statistics

Data were analysed using a one-way ANOVA. Differences were considered significant at $p<0.05$.

Results

Transfection efficiency

First, we tested the efficacy and efficiency of our transfection protocol. Transfected and non-transfected mice were injected intraperitoneally with luciferin, and luciferase activity was visualised using a Night-OWL bioluminescence camera. As shown in Figure 1, non-transfected mice had no luminescence in the gastrocnemius muscles following injection with luciferin (Figure 1, mice 1 and 2); in contrast, transfected mice had robust luminescence at the site of transfection (Figure 1, mice 3-5). Staining for VSV (to measure the VSV-tagged sFLT-1 protein) confirmed the presence of exogenous sFLT-1 in the renal vasculature (data not shown).

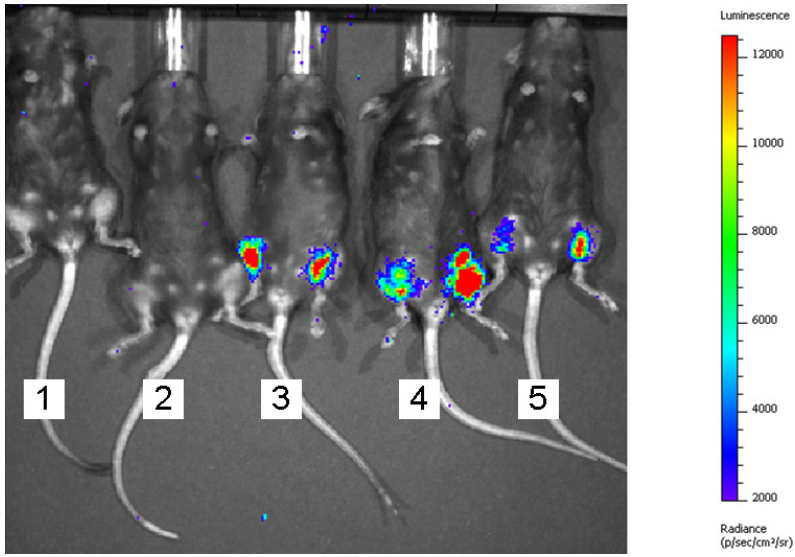

Figure 1: Transfection efficiency. The *sFlt-1* and luciferase constructs were co-transfected bilaterally by injection into the gastrocnemius muscles. Luciferase activity was visualised using a Night-OWL bioluminescence camera and is depicted using an arbitrary pseudocolour scale with red and blue corresponding to high and low luminescence, respectively. 1 and 2: two representative non-transfected *APOC1*-tg mice following an i.p. injection of luciferin; 3-5: three representative *sFlt-1*–transfected *APOC1*-tg mice following an i.p. injection of luciferin.

Albuminuria and glomerulosclerosis

APOC1-tg mice and age-matched wild-type mice have a similar albumin:creatinine ratio (Figure 2A). Moreover, *APOC1*-tg mice that were transfected with *sFlt-1* had a similar albumin:creatinine ratio as non-transfected *APOC1*-tg mice. Furthermore, the glomerular surface area was similar between *APOC1*-tg mice and wild-type mice (Figure 2B), and transfection with *sFlt-1* did not significantly affect glomerular surface area. Non-transfected *APOC1*-tg mice had significantly fewer podocytes compared to wild-type mice, and transfecting *APOC1*-tg mice with *sFlt-1* had no further effect on the number of podocytes (Figure 2C). No other changes in glomerular histology were observed (data not shown). These data indicate that transfecting *APOC1*-tg mice with *sFlt-1* does not accelerate the development of glomerulosclerosis.

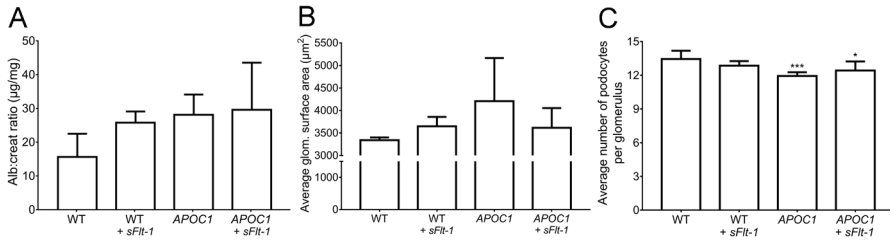

Figure 2: Overexpressing *sFlt-1* in *APOC1*-tg mice for 15 weeks does not cause albuminuria or glomerulosclerosis. At 8 weeks of age, wild-type (WT) and *APOC1*-tg mice were transfected with *sFlt-1*; 15 weeks later, the albumin:creatinine ratio (A), glomerular surface area (B), and number of podocytes per glomerulus (C) were measured. In this and subsequent figures, summary data are presented as the mean ± the standard deviation. *p<0.05 and ***p<0.001 versus the respective wild-type group, one-way ANOVA.

Endothelial cell activation

Glomerular VCAM-1 protein levels were similar between *APOC1*-tg mice and wild-type mice (Figure 3A). In contrast, glomerular ICAM-1 protein levels were significantly higher in the *APOC1*-tg mice compared to wild-type mice (Figure 3B); however, transfection with *sFlt-1* had no effect on either glomerular VCAM-1 or glomerular ICAM-1 protein levels compared to the respective non-transfected groups. Lastly, glomerular *Vegf-a* mRNA levels were similar between all four groups (Figure 3C).

sFlt-1–transfected *APOC1*-tg mice have fewer glomerular macrophages compared to non-transfected *APOC1*-tg mice

Consistent with our previous findings[5], we found that *APOC1*-tg mice have significantly more glomerular macrophages compared to wild-type mice (Figure 4). Interestingly, however, we found that *APOC1*-tg mice transfected with *sFlt-1* have significantly fewer glomerular macrophages compared to non-transfected *APOC1*-tg mice; 15 weeks after transfection, the number of glomerular macrophages was reduced to wild-type levels (Figure 4).

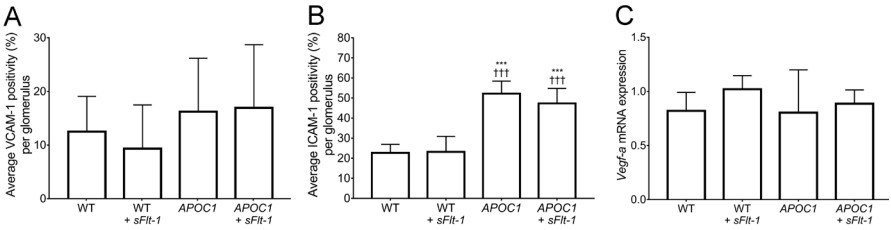

Figure 3: ***APOC1*-tg mice have increased glomerular endothelial cell activation**. *APOC1*-tg and wild-type mice were treated as in Figure 2, and glomerular VCAM-1 (A), ICAM-1 (B), and *Vegf-a* mRNA (C) were measured 15 weeks after transfection. ***$p<0.001$ versus wild-type mice and $^{\dagger\dagger\dagger}p<0.001$ versus *sFlt-1*–transfected wild-type mice, one-way ANOVA.

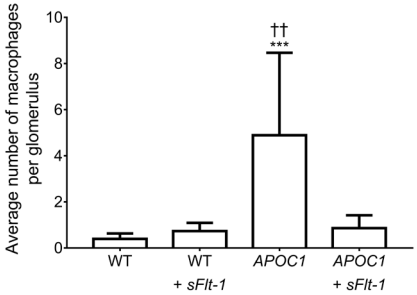

Figure 4: **Expressing *sFlt-1* in *APOC1*-tg mice reduces the glomerular infiltration of macrophages to wild-type levels**. *APOC1*-tg and wild-type mice were treated as in Figure 2, and the number of glomerular macrophages was counted 15 weeks after transfection. ***$p<0.001$ versus non-transfected wild-type mice and $^{\dagger\dagger}p<0.01$ versus both *sFlt-1*-transfected wild-type mice and *sFlt-1*-transfected *APOC1*-tg mice, one-way ANOVA.

Atopic dermatitis is resolved in *APOC1*-tg mice following transfection with *sFlt-1*

Consistent with a previous report[13], *APOC1*-tg mice developed atopic dermatitis, with increased thickness of the epidermal layer compared to wild-type mice (Figure 5). Interestingly, these lesions were not present in the *APOC1*-tg mice that were transfected with *sFlt-1* (Figure 5C, F). The quantification of the thickness of the epidermal layer is provided in Figure 5G. In addition, we found that inflammatory cells were present in the skin of *APOC1*-tg mice (Figure 5H), including macrophages (Figure 5I) and few T cells (Figure 5J). In contrast,

inflammatory cells were not present in the skin of *sFlt-1*–transfected *APOC1*-tg mice (Figure 5F).

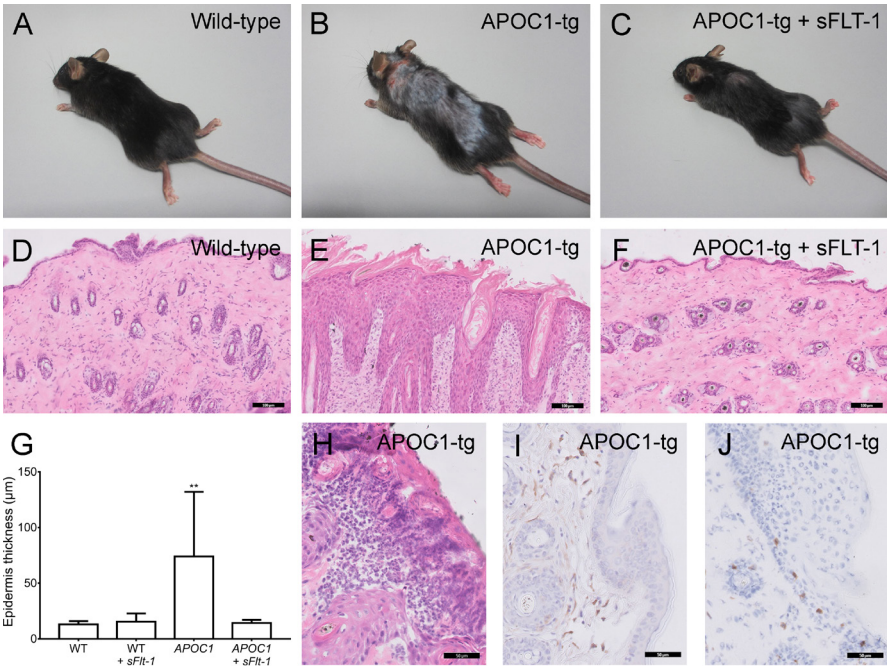

Figure 5: Transfecting *APOC1*-tg mice with *sFlt-1* prevents atopic dermatitis. Representative images of the skin of a wild-type mouse (A, D), an *APOC1*-tg mouse (B, E, H-J), and an *sFlt-1*–transfected *APOC1*-tg mouse (C, F). (G) Summary of epidermal thickness measured in the indicated groups. The sections shown in D-F and H were stained with H&E. Example images of F4/80 (I) and CD3 (J) immunohistochemical staining in skin sections from an *APOC1*-tg mouse. The scale bars represent 100 µm (D-F) and 50 µm (H-J). $^{**}p<0.01$ versus all three other groups, one-way ANOVA.

Discussion

APOC1-tg mice develop nodular glomerulosclerosis by the age of 15 months[5], making them poorly suited for use in therapeutic or preventive intervention studies. Therefore, we attempted to accelerate the development of glomerulosclerosis by transfecting these mice with *sFlt-1* at 8 weeks of age; we then looked for signs of glome-

rulosclerosis 15 weeks after transfection. To our surprise, *APOC1*-tg mice transfected with *sFlt-1* did not develop glomerulosclerosis within the investigated time frame; instead, these mice had fewer glomerular macrophages compared to non-transfected *APOC1*-tg mice, and they showed no signs of atopic dermatitis.

VEGF-A plays a key role in the health and function of endothelial cells, controlling their migration, proliferation, and survival[8]. Decreased levels of glomerular VEGF-A result in damage to the glomerular endothelium; specifically, homozygous glomerular VEGF-A knockout mice are not viable, present with a wide range of glomerular endothelial defects, and fail to develop a glomerular filtration barrier[9]. Moreover, diabetic mice that are heterozygous for VEGF-A selectively in podocytes have increased proteinuria, mesangial matrix expansion, and increased glomerular cell death compared to wild-type diabetic mice[14]. Furthermore, deleting VEGF-A in the kidneys in an inducible VEGF-A knockout mouse induces vascular damage by promoting thrombotic microangiopathy[15]. Similarly, increasing the serum levels of sFLT-1, a decoy receptor for VEGF-A, causes hypertension, glomerular endotheliosis, and proteinuria in rats[10]. In patients with kidney cancer, breast cancer, or colorectal cancer, treatment with an anti-VEGF-A antibody in order to inhibit tumour angiogenesis leads to proteinuria in a dose-dependent manner[16]. Finally, treating wild-type mice with either anti-VEGF-A antibodies or sFLT-1 causes a loss of glomerular endothelial cells, glomerular hypertrophy, and proteinuria[17]. Taken together, these findings indicate that decreasing glomerular VEGF-A levels with sFLT-1 results in glomerular endothelial damage and subsequent glomerular disease. Therefore, we hypothesised that transfecting mice with *sFlt-1* would lead to a systemic increase in sFLT-1, thereby affecting the health and function of glomerular endothelial cells, consequently accelerating the progression of glomerulosclerosis in *APOC1*-tg mice. However, we found that transfecting 8-week-old *APOC1*-tg mice with *sFlt-1* did not lead to the development of glomerulosclerosis, even after 15 weeks. Furthermore, similar to a previous report[13], *APOC1*-tg mice develop atopic dermatitis, a skin disease characterised by scaling, lichenification, excoriations, and pruritus, with thickening of the epidermis and influx of inflammatory cells. The majority of these inflammatory cells were macrophages, although a

few T cells were also present (Figure 5I-J). Interestingly, however, atopic dermatitis was resolved in *sFlt-1*-transfected *APOC1*-tg mice, resulting in normal epidermal thickness and a lack of inflammatory cells in the skin (Figure 5). We previously reported that type 1 diabetic mice transfected with *sFlt-1* have fewer glomerular macrophages, normal glomerular morphology, and improved renal function compared to non-transfected type 1 diabetic mice[12].

One possible explanation for the lack of accelerated glomerulosclerosis in *sFlt-1*–transfected *APOC1*-tg mice may be that the systemic levels of sFLT-1 were insufficient to induce glomerular endothelial damage within the investigated time frame; in other words, glomerular VEGF-A levels may have remained within physiological levels, thereby maintaining the health and function of glomerular endothelial cells. On the other hand, the higher levels of sFLT-1 may have affected other VEGF-A targets aside from its effect on endothelial cells, for example by affecting VEGF-A–induced migration of monocytes and/or macrophages[18,19], which is mediated by the binding of VEGF-A to the FLT-1 receptor on these cells[19]. This possible reduction in cell migration may be the result of VEGF-A sequestering by sFLT-1. In addition, VEGF-A can induce the activation of endothelial cells[12,20]; however, the observed increase in glomerular endothelial cell activation (measured as an increase in glomerular ICAM-1 levels) in *APOC1*-tg mice was not reduced by transfecting *APOC1*-tg mice with *sFlt-1*. Finally, glomerular *Vegf-a* mRNA levels were similar between *APOC1*-tg mice and wild-type mice. Taken together, these results suggest that VEGF-A does not appear to play a role in the increase in glomerular macrophages in *APOC1*-tg mice.

Interestingly, Jin *et al.* recently reported that sFLT-1 has other functions in addition to sequestering VEGF-A[21]. Specifically, sFLT-1 plays an essential role in podocyte cell morphology and glomerular barrier function – independent of VEGF-A – by binding directly to the glycosphingolipid monosialodihexosylganglioside (GM3) in lipid rafts on the surface of podocytes. Monocytes also express GM3, and this expression is increased upon differentiation of monocytes into macrophages and during inflammatory responses[22,23]. Therefore, sFLT-1 may bind to monocytes/macrophages in a similar fashion as sFLT-1 binds to podocytes (i.e. via GM3), subsequently altering cellular function.

This notion is supported by the finding that sFLT-1 downregulates the FLT-1 receptor in leukocytes by decreasing the activity of the *FLT-1* promoter, thereby preventing subsequent migration of these cells upon stimulation with VEGF-A[24]; thus, the reduction in monocyte/macrophage migration with sFLT-1 may be independent of VEGF-A sequestering. Preliminary *in vitro* data by our group suggest that sFLT-1 does indeed affect monocyte/macrophage function. For example, monocytes that differentiate into macrophages in the presence of sFLT-1 develop a different morphology than monocytes that differentiate in the absence of sFLT-1; specifically, the former are more circular in shape and have fewer – or no – filopodia. In addition, the cytokine expression pattern in macrophages that are activated with lipopolysaccharide (LPS) differs depending on whether the macrophages were differentiated in the presence or absence of sFLT-1; specifically, LPS-activated macrophages that were differentiated in the presence of sFLT-1 have reduced expression of several inflammation-related proteins, including interleukin-6 and TNF-alpha (our unpublished data). Taken together, these data suggest that transfecting *APOC1*-tg mice with *sFlt-1* reduces the migration of monocytes by downregulating *FLT-1* promoter activity in these cells, potentially affecting the cells' differentiation into macrophages, thereby reducing both atopic dermatitis and the number of glomerular macrophages.

Similar results have been reported by groups that investigated the use of sFLT-1 to treat a variety of other conditions, including vascular disease[25,26], arthritis[27,28], sepsis[29], and psoriasis[30]; specifically, these studies showed that sFLT-1 was beneficial in terms of reducing both systemic and local inflammation, as well as reducing the number of macrophages at the site of pathology.

In summary, we report that transfecting *APOC1*-tg mice with *sFlt-1* to increase systemic sFLT-1 levels does not accelerate the development of glomerulosclerosis in the investigated time frame; rather, this treatment significantly reduces the number of glomerular macrophages and completely resolves the atopic dermatitis that normally develops in *APOC1*-tg mice. Future studies are needed in order to determine whether these effects of sFLT-1 treatment are dependent upon VEGF-A or whether sFLT-1 has other immunosuppressing properties that are independent of its effect on VEGF-A.

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