Complement C5a induces mesenchymal stem cell apoptosis during the progression of chronic diabetic complications

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Abstract

Aims/hypothesis Regeneration and repair mediated by mesenchymal stem cells (MSCs) are key self-protection mechanisms against diabetic complications, a reflection of diabetes-related cell/tissue damage and dysfunction. MSC abnormalities have been reported during the progression of diabetic complications, but little is known about whether a deficiency in these cells plays a role in the pathogenesis of this disease. In addition to MSC resident sites, peripheral circulation is a major source of MSCs that participate in the regeneration and repair of damaged tissue. Therefore, we investigated whether there is

a deficiency of circulating MSC-like cells in people with diabetes and explored the underlying mechanisms.

Methods The abundance of MSC-like cells in peripheral blood was evaluated by FACS. Selected diabetic and non-diabetic serum (DS and NDS, respectively) samples were used to mimic diabetic and non-diabetic microenvironments, respectively. The proliferation and survival of MSCs under different serum conditions were analysed using several detection methods. The survival of MSCs in diabetic microenvironments was also investigated in vivo using leptin receptor mutant (*Lep^{db/db}*) mice.

Results Our data showed a significant decrease in the abundance of circulating MSC-like cells, which was correlated with complications in individuals with type 2 diabetes. DS strongly impaired the proliferation and survival of culture-expanded MSCs through the complement system but not through exposure to high glucose levels. DS-induced MSC apoptosis was mediated, at least in part, by the complement C5a-dependent upregulation of Fas-associated protein with death domain (FADD) and the Bcl-2-associated X protein (BAX)/B cell lymphoma 2 (Bcl-2) ratio, which was significantly inhibited by neutralising C5a or by the pharmacological or genetic inhibition of the C5a receptor (C5aR) on MSCs. Moreover, blockade of the C5a/C5aR pathway significantly inhibited the apoptosis of transplanted MSCs in *Lep^{db/db}* recipient mice.

Conclusions/interpretation C5a-dependent apoptotic death is probably involved in MSC deficiency and in the progression of complications in individuals with type 2 diabetes. Therefore, anticomplement therapy may be a novel intervention for diabetic complications.

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Keywords Apoptosis · C5a/C5aR pathway · Complement · Complication · Deficiency · Mesenchymal stem cell · Type 2 diabetes

Abbreviations

BAX	Bcl-2-associated X protein
Bcl-2	B cell lymphoma 2
C5aR	C5a receptor
CH50	50% complement haemolytic activity
CI	Cell index
DS	Diabetic serum
FADD	Fas-associated protein with death domain
FPG	Fasting plasma glucose
HI-DS	Heat-inactivated diabetic serum
HI-NDS	Heat-inactivated non-diabetic serum
hUC-MSC	Human umbilical cord-derived mesenchymal stem cell
MAC	Membrane attack complex
mBM-MSC	Mouse bone marrow-derived mesenchymal stem cell
MSC	Mesenchymal stem cell
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NDS	Non-diabetic serum
PBMC	Peripheral blood mononuclear cell
PB-MSC	Peripheral blood-derived mesenchymal stem cell
RTCA	Real-time cell analysis
sC5b-9	Soluble C5b-9 complex
WT	Wild-type

Introduction

The global prevalence of diabetes has nearly doubled since 1980, increasing from 4.7% to 8.5% in the adult population, and an estimated 422 million adults were living with diabetes in 2014 [1, 2]. The number of people with diabetes is estimated to increase to 552 million worldwide by 2030 [3], and one in ten adults will have diabetes by 2040 [4]. As a major cause of death and disability worldwide, diabetes places a heavy burden on individuals, families and the healthcare system [1, 4–6].

People with diabetes have a high risk of developing a series of related complications, including diabetic cardiomyopathy, retinopathy, neuropathy, foot ulcers and nephropathy [7]. The risk factors and mechanisms involved in the pathogenesis of complications in individuals with diabetes are complex and have not been totally defined [8, 9]; thus, the prevention, treatment and reversal of diabetic complications are restricted.

Diabetic complications are a reflection of metabolic disorder-induced cell/tissue damage and dysfunction, with manifestations in multiple organs. In view of the essential roles of mesenchymal stem cells (MSCs) in target tissue regeneration and repair [10–13], we may reasonably speculate that there must be quantitative, distributive or functional anomalies in endogenous MSCs in individuals with diabetes

who present complications that impair the ability of these cells to regenerate and repair the damaged tissue. Indeed, a pathogenic role for impaired MSC quality in diabetes has been proposed by many researchers [14–18], and a decreased number of MSCs has also been reported to be associated with this disease [19, 20]; however, the causes and role of MSC deficiency in diabetes are unknown.

When tissue is damaged, resident MSCs, which rapidly function as seed cells, are recruited to the injury site and a large number of MSCs are also recruited from the peripheral circulation to participate in the regeneration and repair of the injured tissue [18, 19]. Therefore, we speculate that the number of circulating MSCs in individuals with diabetes might be decreased, ultimately resulting in an insufficient number of these cells at the injury site and the impaired regeneration and repair of target tissues. Moreover, both hyperglycaemia and excessive complement activation are characteristics of diabetes that have been reported to be involved in the pathogenesis of diabetic complications [9, 21–24]; thus, we investigated their correlations with the abundance of circulating MSCs in this study.

Methods**Ethics statement**

All work was conducted in full compliance with ethical principles, and prior approval was obtained from the Ethics Committee of Daping Hospital, Third Military Medical University (Chongqing, China). Human samples were collected after obtaining informed consent from the donors or their legal representatives, and the mouse experiments conformed to ethical standards.

Participants and serum collection

Individuals with type 2 diabetes and age- and sex-matched healthy donors were recruited from Daping Hospital. The demographic and clinical data are provided in Table 1 and Electronic Supplementary Material (ESM) Table 1. Serum samples from all participants were collected and stored according to the principles proposed by Lachmann [25] (see ESM [Methods](#) for details).

Genetically modified mice

Genetic mouse models of diabetes B6.BKS(D)-*Lepr*^{db}/J (Jackson Laboratory, Bar Harbor, ME, USA) and C5a receptor (C5aR)-deficient (*Cd88*^{−/−}) mice (*C5ar1*^{tm1Cge}; BALB/c background; Jackson Laboratory; please note *Cd88* is an alias of *C5ar1*) were kindly provided by Y. Wang (Department of Medical Genetics, Third Military Medical University) and G.

Table 1 Demographic and clinic characteristics of the diabetic and control participants

Characteristic	Control group (<i>n</i> = 15)	Type 2 diabetes group (<i>n</i> = 49)			<i>p</i> value
		0 complication (<i>n</i> = 14)	1 complication (<i>n</i> = 19)	≥2 complications (<i>n</i> = 16)	
Age (years)	63.5 ± 5.2	62.2 ± 5.8	63.4 ± 4.5	65.6 ± 4.1	0.290
Sex (male/female)	9/6	8/6	11/8	9/7	0.997
BMI (kg/m ²)	23.3 ± 1.7	25.9 ± 1.7**	24.6 ± 1.6*	23.8 ± 2.3	0.002
Duration of diabetes (years)	NA	3.3 ± 1.3	8.7 ± 1.7 [†]	15.2 ± 3.6 [†]	<0.001
FBG (mmol/l)	4.6 ± 0.6	8.1 ± 0.6***	8.8 ± 1.2***	9.9 ± 1.3***	<0.001
HbA _{1c} (%)	4.5 ± 0.8	7.7 ± 0.7***	8.5 ± 0.5***	9.1 ± 0.9***	<0.001
HbA _{1c} (mmol/mol)	25.5 ± 8.3	60.8 ± 7.6	69.1 ± 5.5	75.8 ± 10.1	

Data are presented as means ± SD

All data (excluding sex) were analysed using one-way ANOVA, followed by Dunnett's test for multiple comparisons; sex was analysed using χ^2 .

* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with control participants; [†] $p < 0.001$ compared with diabetic individuals with no complications

NA, not applicable

Xu (Institute of Immunology, College of Basic Medical Science, Third Military Medical University), respectively. For details regarding housing, genotyping, breeding methods and the use of control mice, see ESM [Methods](#).

Quantitative detection of MSC-like cells in peripheral blood

Approximately 4 ml of anticoagulated blood was harvested to analyse the cell subgroups. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation within 2 h of sample collection. Then, the proportion and number of MSC-like (CD34[−]CD105⁺) cells among PBMCs were determined by FACS analysis. Experimenters were blind to group allocation and outcome assessment. See ESM [Methods](#) for details.

For similar detection in mice, anticoagulant peripheral blood was resuspended in commercial ACK lysis buffer (Beyotime, Haimen, China) to lyse the erythrocytes. Then, the nucleated cells were stained and analysed to determine the abundance of MSC-like cells.

Isolation and culture of MSCs

Human umbilical cord-derived MSCs (hUC-MSCs) were isolated using the tissue block implantation method (see ESM [Methods](#) for details). For subcultures of hUC-MSCs, the culture medium was replaced with DMEM/F12 (HyClone, Logan, UT, USA) containing 10% FBS (Gibco, Grand Island, NY, USA).

Mouse bone marrow-derived MSCs (mBM-MSCs) were isolated using the simple plastic adherent method (see ESM [Methods](#) for details).

In this study, hUC-MSCs were used between the second and fifth passages, and mBM-MSCs were used between the

fourth and sixth passages. The MSCs were identified by their morphology, surface markers and multi-directional differentiation ability (ESM Fig. 1; see ESM [Methods](#) for details).

Cell viability assay

When the inoculated hUC-MSCs in 96-well plates had attached to the bottom of the plates or were in the mid-logarithmic growth phase, the medium was replaced with DMEM/F12 containing different concentrations of D-glucose (Sigma-Aldrich, St Louis, MO, USA) or different types of sera. After treatment for the indicated period, cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and the relative cell viability was calculated. See ESM [Methods](#) for details.

Determination and quantification of apoptotic death

TUNEL method Single-cell suspensions of hUC-MSCs were seeded on coverslips in a 12-well plate. During the mid-logarithmic growth phase, the cells were rinsed and further incubated under different culture conditions. After 12 h, the hUC-MSCs on the coverslips were fixed with 4% paraformaldehyde (PFA), permeabilised with Triton X-100, stained using a TUNEL Andy Fluor 488 Apoptosis Detection Kit (GeneCopoeia, Guangzhou, China), and further stained with DAPI (Sigma-Aldrich). After being washed with PBS, the cells on coverslips were photographed using a fluorescence microscope (Olympus IX71, Tokyo, Japan).

FACS analysis MSCs in mid-logarithmic growth phase in six-well plates were rinsed and incubated under different culture conditions for 12 h. Then, both the adherent and suspended cells were collected and double stained with annexin V-FITC/PI (KeyGen Biotech, Nanjing, China). Cell

apoptosis was measured within 30 min by FACS. Both early (annexin V-FITC⁺PI⁻) and late (annexin V-FITC⁺PI⁺) apoptotic cells were included in the cell death analyses.

Real-time cell analysis A real-time cell analysis (RTCA) system (ACEA Biosciences, Hangzhou, China) that can dynamically measure impedance-based signals and present the signals as cell index (CI) values was used to continuously monitor the effects of different culture conditions on the adherence and proliferation of hUC-MSCs. See ESM [Methods](#) for details.

Complement haemolytic activity and serum levels of complement components

Human serum samples were thawed rapidly from -196°C , and the 50% complement haemolytic activity (CH50) was determined using the red cell haemolysis test (see ESM [Methods](#) for details). The levels of complement C3 and C4 were measured by technicians at the Department of Clinical Laboratory, Daping Hospital, using the immunoturbidimetry method. The soluble C5b-9 complex (sC5b-9) and C5a were assessed in an enzyme immunoassay (EIA) using the MicroVue C5a EIA and sC5b-9 Plus EIA kits (Quidel; San Diego, CA, USA) according to the manufacturer's instructions.

Western blotting analysis

MSCs were seeded into 60 mm dishes and treated with different types of sera when they reached 70% confluence. After 12 h, the total proteins were extracted, quantified, denatured, separated, immunoblotted and visualised. See ESM [Methods](#) and ESM Table 2 for details.

Immunocytochemical analysis

PE-conjugated anti-human and FITC-conjugated anti-mouse CD88 (C5aR1) antibodies (1:50 dilutions; Cedarlane; Hornby, ON, Canada) were used to identify and locate CD88 in hUC-MSCs and mBM-MSCs, respectively. See ESM [Methods](#) for details.

Labelling, implantation and detection of mBM-MSCs

Isolation of mBM-MSCs was performed using wild-type (WT) and *Cd88*^{-/-} BALB/c mice (male; 4–6 weeks old). WT and *Lepr*^{db/db} C57BL/6 mice (male; 8 months old) were used to evaluate the effects of diabetes and the C5a/C5aR pathway on the survival of mBM-MSCs in vivo. Briefly, pre-labelled mBM-MSCs were implanted into WT or *Lepr*^{db/db} mice and their survival was detected by FACS. See ESM [Methods](#) for details.

Data analysis

GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA) was used for data analysis. Data are expressed as means $\pm$ SD or means $\pm$ SEM as indicated. Comparisons between groups were analysed using *t* tests (two-sided; unpaired) or ANOVA. Linear regression and Pearson's correlation analyses were performed to determine whether a relationship existed between two variables. A *p* value of <0.05 was considered to be statistically significant.

Results

Numbers of circulating MSC-like cells are decreased in individuals with diabetes and in *Lepr*^{db/db} mice

The average numbers (mean [range]) of CD34⁺CD105⁺ MSC-like cells present in 1×10^5 PBMCs were 338 (154–522) in the healthy control participants, 203 (86–321) in diabetic participants without any complications, 141 (47–272) in individuals with one complication, and 123 (28–231) in individuals with multiple complications (Fig. 1a). These results indicated an obviously lower abundance of circulating MSC-like cells in the participants with diabetes. Furthermore, we divided the participants with diabetes into five groups according to the number of complications they presented and calculated the average number of circulating MSC-like cells for each group. The results revealed a negative correlation between the number of complications and the abundance of circulating MSC-like cells (Fig. 1b). Those with a longer duration of diabetes or a higher fasting plasma glucose (FPG) levels also tended to have a lower abundance of circulating MSC-like cells (Fig. 1c,d). The lower abundance of these cells was confirmed in *Lepr*^{db/db} mice (Fig. 1e).

DS impairs the proliferation and survival of hUC-MSCs in vitro

We evaluated the effects of the diabetic microenvironment on the fate of MSCs in vitro to explore the mechanisms underlying the diabetes-related lower abundance of circulating MSC-like cells. As predicted, DS (randomly selected serum samples from individuals with multiple diabetic complications) significantly impaired the proliferation and survival of hUC-MSCs compared with NDS (serum samples from age- and sex-matched healthy control participants) (Fig. 2a, b). The cell cycle progression and apoptosis in hUC-MSCs stimulated by the different serum treatments were further assessed, and we found that DS induced cell cycle arrest (ESM Fig. 2) and apoptotic cell death (Fig. 2c–g).

Fig. 1 Quantitative analysis of the number of circulating MSC-like (CD34⁺CD105⁺) cells and correlation with the number of diabetic complications, duration of diabetes and FPG levels. **(a)** Numbers of circulating MSC-like cells among 1×10^5 PBMCs in diabetic and control participants. **(b)** Analysis of the correlation between the number of complications and the average number of circulating MSC-like cells in individuals with diabetes. **(c, d)** Linear regression analysis of the number of circulating MSC-like cells and duration of diabetes **(c)** or FPG levels **(d)**. **(e)** Numbers of circulating MSC-like cells in *Lepi^{db/db}* and control mice ($n = 5$ for each group). * $p < 0.05$, *** $p < 0.001$

High glucose levels promote hUC-MSC proliferation but have little effect on cell survival

We assessed the proliferation and survival of hUC-MSCs cultured under different concentrations of D-glucose to investigate whether the diabetes-related MSC deficiency is attributed to high glucose levels. The results showed that high glucose levels promoted the proliferation and cell cycle progression of hUC-MSCs (Fig. 3a, b and ESM Fig. 3) and slightly increased cell apoptosis, but only when the concentration reached 30 mmol/l (Fig. 3c, d), which is a relatively high blood glucose level that is not typically observed in most diabetic individuals. We adjusted the glucose concentration in NDS to the concentration detected in DS by adding D-glucose and then compared the apoptosis of hUC-MSCs treated with NDS, DS or adjusted NDS to further exclude a role for high glucose levels in the pro-apoptotic effect of DS. The increase in the glucose concentration in NDS did not increase its cytotoxicity. However, DS substantially increased apoptosis compared with adjusted NDS, although these serum samples contained equal glucose concentrations (Fig. 3e, f). Thus, DS must have contained other anomalous factors in addition to high glucose levels that contributed to its cytotoxicity towards hUC-MSCs in vitro.

Complement C5a mediates the pro-apoptotic effect of DS on cultured hUC-MSCs

It has been repeatedly confirmed that uncontrolled serum complement activation plays an important role in the

pathogenesis of diabetic complications [21–23], while our data (Fig. 1a, b) suggested that fewer circulating MSC-like cells may result in more complications. Hence, we speculated that there may be a correlation between the activation status of the complement system and the abundance of circulating MSC-like cells in diabetes. The CH50 and complement (C3, C4, sC5b-9 and C5a) levels in DS were significantly higher than those in NDS (Table 2), and the abundance of circulating MSC-like cells was negatively correlated with the serum complement activity (ESM Fig. 4). However, a correlation does not imply causality. We then inactivated complement by heating the serum at 56°C for 30 min to determine whether complement played a causal role in mediating the cytotoxicity of DS. The cytotoxic effect of DS on the hUC-MSCs was largely attenuated by the pre-inactivation procedure (Fig. 4a–d). Although the cytotoxicity of DS was much higher than that of NDS, there was no significant difference in the cytotoxicity or pro-apoptotic effect between the heat-inactivated DS (HI-DS) and heat-inactivated NDS (HI-NDS) (Fig. 4a–d).

Typically, complement-dependent cytotoxicity occurs upon assembly of the membrane attack complex (MAC) and its insertion into the plasma membrane of target cells [26]. However, by pre-incubating hUC-MSCs with recombinant human CD59 (rhCD59; Sino Biological, Beijing, China) to inhibit the assembly and insertion of the MAC, we identified only a slight but significant decrease in the DS-induced apoptosis of hUC-MSCs (Fig. 4e, f), suggesting that the MAC may not play a major role in this process. In addition, a large amount of anaphylatoxins will be simultaneously generated

Fig. 2 The effects of NDS or DS on the proliferation and survival of hUC-MSCs. **(a)** The relative cell viability of hUC-MSCs exposed to different sources of sera for 12 h was determined by MTT assay. DS- and NDS-1–4: numbered serum samples (randomly selected) from four diabetic and four control participants, respectively. Data are expressed as means $\pm$ SEM ($n = 5$). **(b)** The adherent areas of hUC-MSCs were continuously monitored by RTCA. Different types of stimulating sera were administered at the indicated time point. Data are expressed as means $\pm$ SD ($n = 2$). Apoptosis of hUC-MSCs stimulated with the different serum treatments for 12 h was detected by **(c, d)** FACS following annexin V/PI double staining ($n = 3$) and **(e, f)** the TUNEL assay. **(e)** Representative photomicrographs of

TUNEL staining. Scale bar, 200 μ m. **(f)** Quantitative evaluation of data from TUNEL assays in which the effects of four independent DS samples and four control NDS samples were evaluated (with three replications). **(g)** The expression of cleaved caspase-3 and poly-ADP ribose polymerase (PARP) in hUC-MSCs exposed to different types of sera for 12 h was determined by western blotting. The numbers under each blot represent the relative quantification of the band density, which were calculated as the ratio of the band density of the FBS group or DS group to the band density of the NDS group. Data are from assays in which the effects of four independent DS samples and four control NDS samples were evaluated and are presented as means. * $p < 0.05$, *** $p < 0.001$

during complement activation. Of these anaphylatoxins, C5a is the most potent in eliciting biological responses [27, 28], which seems to be important for inducing cell apoptosis [29–32]. As shown in Fig. 4e, f and ESM Fig. 5a, the pro-apoptotic effect of DS was significantly decreased when it was pre-treated with an anti-C5a antibody (Abcam, Cambridge, UK; 10 μ g/ml for 30 min) compared with untreated DS. In addition, the addition of recombinant human C5a (rhC5a; Sino Biological) to HI-DS, which lacks complement activity, partially rescued the pro-apoptotic effect. However, although C5a acts as a critical mediator of the cytotoxicity of DS against hUC-MSCs, C5a alone did not affect the survival and proliferation of hUC-MSCs over the tested concentration range (0–10 μ mol/l) (Fig. 4e, f and ESM Fig. 5).

The C5a/C5aR pathway plays an essential role in DS-induced apoptosis of MSCs

C5a exerts its effects through C5aRs, i.e. C5aR1 (CD88) and C5a receptor-like 2 (C5L2). CD88 mediates signalling activity, whereas C5L2 is generally termed a decoy receptor and its function is still enigmatic and controversial [33]. We detected the positive expression of CD88 in both hUC-MSCs and mBM-MSCs (Fig. 5a) and further confirmed its location on the surfaces of these cells (Fig. 5b). Moreover, DS induced an increase in CD88 expression in hUC-MSCs (Fig. 5c), indicating that the C5a/C5aR pathway had been activated in the DS-exposed hUC-MSCs. BM-MSCs derived from *Cd88*^{−/−} mice were assessed to determine the role of this pathway in DS-induced apoptosis. We

Fig. 3 Effects of different concentrations of D-glucose on the proliferation and survival of hUC-MSCs. **(a)** The relative cell viability of hUC-MSCs exposed to different concentrations of D-glucose for 12 h was determined by MTT assay ($n = 5$). **(b)** The effects of different concentrations of D-glucose on the proliferation and viability of hUC-MSCs were continuously monitored by RTCA ($n = 2$). **(c, d)** The apoptosis of hUC-MSCs exposed to different concentrations of D-glucose for 12 h was detected by FACS following annexin V/PI double staining ($n = 3$). **(e, f)**

The apoptosis of hUC-MSCs that had been treated with FBS, NDS, adjusted NDS or DS for 12 h was determined by FACS with three replications: **(e)** a representative example of results obtained from one set of experiments in which one DS sample and one control NDS sample were used; **(f)** statistical analysis of the results of FACS analyses in which the effects of four independent DS samples and four control NDS samples were evaluated. Control (CTRL), normal culture condition containing 17.5 mmol/l D-glucose. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

did not observe an obvious increase in the apoptosis of *Cd88*^{-/-} mBM-MSCs exposed to DS compared with cells exposed to

NDS (Fig. 5d). In addition, following treatment with DS, the expression of pro-apoptotic proteins (Fas-associated protein

Table 2 Serum levels of CH50, complement components (C3 and C4) and activation products (sC5b-9 and C5a) in diabetic and control participants

Variable	Control group ($n = 15$)	Type 2 diabetes group ($n = 49$)			p value
		0 complication ($n = 14$)	1 complication ($n = 19$)	≥ 2 complications ($n = 16$)	
CH50 (U/ml)	37.55 $\pm$ 6.86	44.25 $\pm$ 9.79	45.48 $\pm$ 14.09	47.46 $\pm$ 14.17	0.12
C3 (g/l)	1.01 $\pm$ 0.16	1.10 $\pm$ 0.10	1.14 $\pm$ 0.17	1.17 $\pm$ 0.22*	0.05
C4 (g/l)	0.31 $\pm$ 0.12	0.38 $\pm$ 0.09	0.40 $\pm$ 0.12	0.41 $\pm$ 0.14	0.11
sC5b-9 (μ g/l)	83.31 $\pm$ 21.50	108.60 $\pm$ 16.51*	121.90 $\pm$ 26.91***	132.40 $\pm$ 29.56***	<0.001
C5a (μ g/l)	6.38 $\pm$ 2.58	14.02 $\pm$ 5.60**	20.61 $\pm$ 7.62***	24.99 $\pm$ 8.90***	<0.001

Data are presented as means $\pm$ SD

All data were analysed using one-way ANOVA, followed by Dunnett's test for multiple comparisons

* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with control participants

Fig. 4 Complement mediates the cytotoxicity of DS towards hUC-MSCs. **(a)** The relative cell viability of hUC-MSCs cultured under different serum conditions for 12 h was determined by MTT assay ($n = 5$). **(b)** The dynamic effects of untreated or heat-inactivated human serum samples on hUC-MSCs were analysed by RTCA ($n = 2$). **(c, d)** The apoptosis of hUC-MSCs exposed to untreated or heat-inactivated sera for 12 h was determined by FACS following annexin V/PI double staining. **(e, f)** The effects of blockade of MAC formation or the biological effects of C5a on the

survival of hUC-MSCs exposed to human serum samples were evaluated using the TUNEL assay. Scale bars, 200 μm. White bars, untreated control; black bars, heat-inactivated samples. **(b, c, f)** Illustrate the representative results obtained from one set of experiments in which one DS sample and one control NDS sample were used; **(d, e)** are statistical analyses of the results of FACS analyses or TUNEL assays in which the effects of four independent DS samples and four control NDS samples were evaluated. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

with death domain [FADD], cleaved caspase-3 and caspase-8) and the Bcl-2-associated X protein (BAX)/B cell lymphoma 2 (Bcl-2) ratio were much lower in $Cd88^{-/-}$ mBM-MSCs than in WT mBM-MSCs (Fig. 5e). We further confirmed the regulatory effects of the C5a/C5aR pathway on the expression of apoptosis-related proteins in hUC-MSCs by antagonising CD88 (a 30 min preincubation with 100 nmol/l PMX-53 [AcF-(OPdChaWR); GL Biochem, Shanghai, China]) or by the addition of rhC5a (Fig. 5f).

Blockade of the C5a/C5aR pathway inhibits MSC apoptosis in vivo

As indicated above, MSC apoptosis in diabetes mellitus might be mediated by the C5a/C5aR pathway. We transplanted prelabelled BM-MSCs from WT or $Cd88^{-/-}$ mice into WT or

$Lepr^{db/db}$ mice and analysed the apoptosis of the transplanted cells to validate this hypothesis in vivo (Fig. 6a). In $Lepr^{db/db}$ recipient mice, the rate of $Cd88^{-/-}$ mBM-MSC apoptosis was much lower than the rate in the WT mBM-MSCs, and apoptosis of the WT mBM-MSCs was significantly inhibited by daily i.p. injections with 1 mg/kg PMX-53 starting on the day before transplantation (Fig. 6b). The rate of WT mBM-MSC apoptosis was much higher in $Lepr^{db/db}$ recipient mice than in the WT recipient mice, whereas the rates of $Cd88^{-/-}$ mBM-MSC apoptosis were similar in the WT and $Lepr^{db/db}$ recipient mice (Fig. 6c).

Discussion

In diabetes mellitus, a variety of cells and tissues are affected primarily from damage or dysfunction induced by chronic

Fig. 5 DS-induced MSC apoptosis depends on the upregulation of FADD and the BAX/Bcl-2 ratio mediated by the C5a/C5aR pathway. **(a)** The level of CD88 in MSCs was determined by western blotting. **(b)** Immunocytochemical detection of CD88 on MSCs. Images display both CD88 (red in hUC-MSCs; green in mBM-MSCs) and the nuclear stain DAPI (blue). Scale bars, 100 μ m. **(c)** The levels of CD88 in hUC-MSCs exposed to NDS or DS. **(d)** The apoptosis of $Cd88^{-/-}$ mBM-MSCs exposed to the different serum treatments for 12 h was determined by FACS following annexin V/PI double staining. Data are presented as

means $\pm$ SEM ($n = 4$). **(e)** The levels of apoptosis-related proteins in WT or $Cd88^{-/-}$ mBM-MSCs exposed to DS for 12 h detected by western blotting. **(f)** The levels of apoptosis-related proteins in hUC-MSCs exposed to the indicated conditions for 12 h measured by western blotting. The numbers under each blot represent the relative quantification of the band density, which were calculated as the ratio of the band density of each group to the band density of the control group. The average gray-scale intensities of control bands are denoted as “1”, and data are presented as means. ** $p < 0.01$, *** $p < 0.001$

hyperglycaemia, and stem cell-dependent regeneration and repair are initiated simultaneously. When regeneration and repair fail to compensate for the damage or dysfunction, complications may arise. MSCs are a population of pluripotent cells with strong capacities for self-renewal, multi-lineage differentiation, paracrine secretion and immunoregulation that are located in virtually all postnatal organs and tissues [34]; thus, these cells have excellent potential for promoting regeneration and repair [10, 35, 36]. In this study, we detected an obvious reduction in the abundance of circulating MSC-like cells in both individuals with type 2 diabetes and diabetic *Lep^{db/db}* mice, and we proposed that the shortage of functional MSCs might promote the development of diabetic complications and that this deficiency is mainly attributed to an increase in complement-induced apoptotic cell death. This proapoptotic process was at least partially mediated by C5a, which activated the extrinsic and intrinsic apoptotic pathways in MSCs upon binding to C5aR (Fig. 6d). We believe that these findings provide insights into the further management and prevention of diabetic complications.

To improve the detection rate of circulating MSCs, we used a haematopoietic marker (CD34) and a mesenchymal marker (CD105) together to identify circulating MSCs. According to the nomenclature and definition of MSC [37, 38], it would be more meaningful if more detection markers were included. Moreover, we explored the underlying mechanisms using

hUC-MSCs and mBM-MSCs instead of peripheral blood-derived MSCs (PB-MSCs), as we failed to culture and expand MSCs from peripheral blood. We believe that the data from PB-MSCs would be more convincing.

Hyperglycaemia is the most prominent characteristic of diabetes, and its effects on the proliferation and survival of MSCs have been repeatedly studied; however, the results of these studies have been contradictory. Two independent teams have reported that high glucose levels induce an increase in MSC apoptosis and a decrease in MSC proliferation [39, 40], whereas two other groups have reported the complete resistance of human MSCs to high glucose toxicity [41, 42]. In addition, Ryu and colleagues have reported that high glucose levels induced MSC proliferation [43]. As shown in our study, high glucose levels promoted hUC-MSC proliferation but did not affect their survival. Nevertheless, the hyperglycaemic condition in diabetes mellitus is chronic and dynamic, whereas the duration of high glucose exposure in our study was no more than 1 week. Therefore, the effects of long-term and fluctuating high glucose levels on the proliferation and survival of MSCs must be studied further.

In the present study, we eliminated the effects of the complement system using a classical heating procedure, but the possibility that some other potential deleterious molecules present in the serum might have been simultaneously inactivated by this heating step cannot be excluded. Moreover, based on the limited

This study has indicated for the first time that excessive activation of the complement system in the diabetic microenvironment not only inhibits MSC proliferation but also induces MSC apoptosis, a process correlated with the progression of diabetic complications. Notably, both individuals with type 2 diabetes and *Lepr^{db/db}* mice are characterised by obesity, a condition in which increased complement activation has

also been reported [48]. However, the diabetic and control participants in this study were not matched for BMI, and thus, we could not be sure of the initial cause of complement activation in participants. It is reasonable to speculate that complement-mediated MSC apoptosis may also occur in obesity, and early weight control may be an effective way to maintain an abundance of circulating MSCs.

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Data availability The datasets generated during the current study are available from the corresponding author upon reasonable request.

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Contribution statement XX and HH conceived/designed the study, reviewed the manuscript and are responsible for the integrity of this work. MZ designed the study, performed the experiments, analysed the data and wrote the manuscript. XH, XHW and WQ performed the experiments and analysed the data. WX, WG, TCA and XTH performed the experiments. LQA and ZL contributed to acquisition of clinical data. XPL and NX made substantial contributions to conception and design of the study and edited the manuscript. JY reviewed/edited the manuscript and contributed to the analysis and interpretation of data. All authors revised the manuscript critically for important intellectual content and approved the final version for publication.

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