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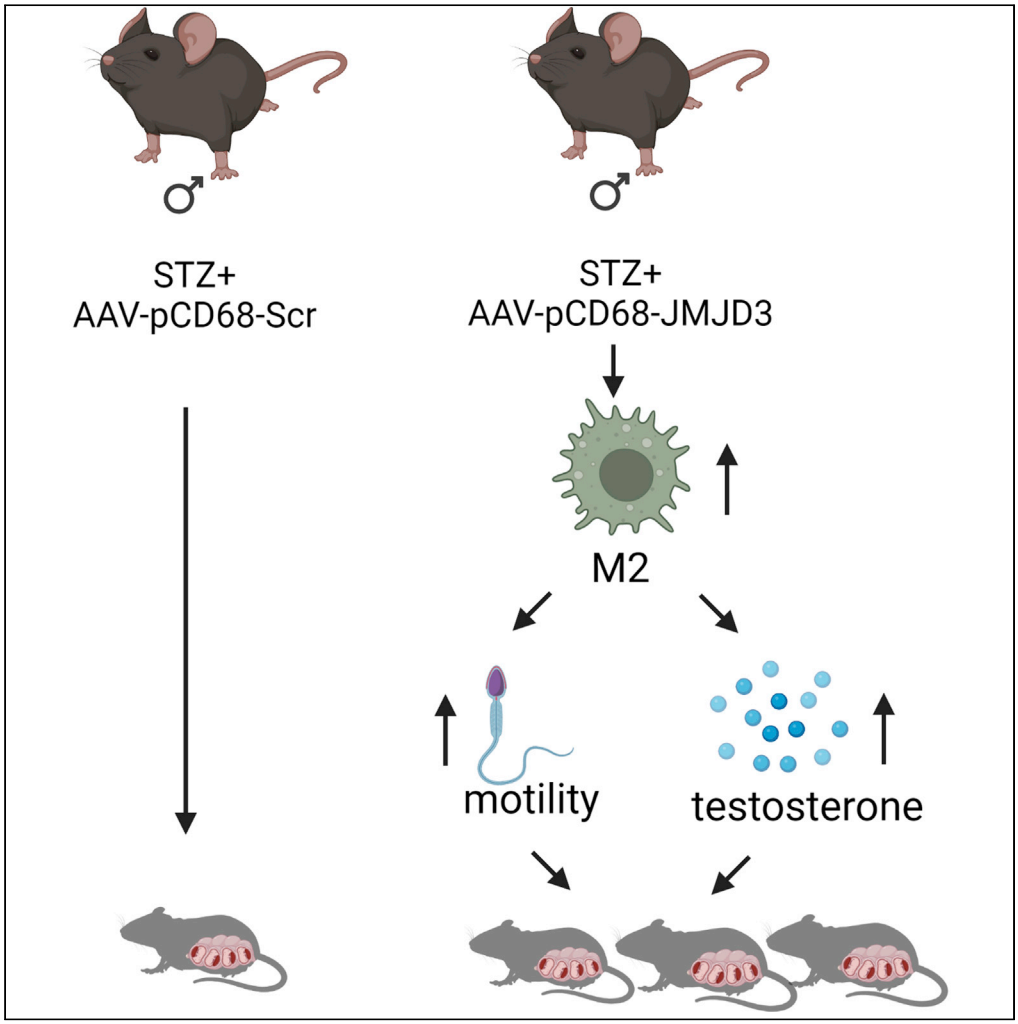

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Highlights

JMJD3-induced M2-polarization of testicular macrophages

M2-polarized testicular macrophages improve mating capability of diabetic male mice

The motility of spermatozoa is alleviated in CD68p-JMJD3-treated diabetic mice

The testosterone levels increase in CD68p-JMJD3-treated diabetic mice

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Alternative polarization of resident macrophages improves hyperglycemia-associated male infertility

Xiaobin Zhu,[1] Zhihong Niu,[1] Weimin Fan,[1] Meiyu Cheng,[1] Qian Chen,[1,*] and Aijun Zhang[1,2,*]

SUMMARY

Recent studies have demonstrated that hyperglycemia induces inflammation in male reproductive system to cause sperm damages and infertility, which may be associated with re-polarization of tissue macrophages from an anti-inflammation M2-like subtype to a pro-inflammation M1-like subtype. However, the underlying mechanisms are not fully determined and a practical approach to interfere with the progression of infertility is lacking. Here, we transduced the testicular macrophages back to the M2-like phenotype with adeno-associated viruses carrying an M2-trigger, Jumonji domain-containing protein D3 (JMJD3), under a macrophage-specific CD68 promoter (CD68p-JMJD3), in streptozotocin-induced diabetic mice. We found that JMJD3-induced M2-polarization of testicular macrophages significantly improved the mating capability of diabetic male mice. The diabetes-induced impairment of the motility of spermatozoa and the decreases in the serum and testicular testosterone levels were both significantly alleviated in CD68p-JMJD3-treated diabetic mice. Thus, our study proposes a practical strategy to treat hyperglycemia-associated infertility.

INTRODUCTION

Diabetes is the most prevalent chronic disease worldwide and it affects the human beings from many aspects (Schaper et al., 2012). Infertility occurs frequently in diabetic male patients (Maresch et al., 2018). In the past, more than 1000 articles have shown strong evidence that higher ratio of infertility is detected in diabetic male than non-diabetic controls (Maresch et al., 2018). However, most of these studies did not investigate the underlying mechanisms, with the very few has shown that hyperglycemia-induced impairment of hypothalamicpituitary-gonadal axis, increases in DNA damage, augmentation in oxidative stress and endoplasmatic reticulum stress in cells, etc, may play roles in the diabetes-associated male infertility (Maresch et al., 2018).

Macrophages play critical roles in a variety of biological events (Li et al., 2018; Olefsky and Glass, 2010). Macrophages are functionally polarized into a pro-inflammatory M1 and an anti-inflammatory M2 cells in response to different stimulants (Mills, 2012). Chronic hyperglycemia has been shown to alter macrophage polarization in different tissue and organs, and the macrophages with phenotypic changes are key players to coordinate the disease progression (Eguchi and Manabe, 2013; Ehses et al., 2008; Fujisaka, 2021; Meshkani and Vakili, 2016; Sunahara and Martins, 2012; Yaseen and Khamaisi, 2020). Recent studies have demonstrated that hyperglycemia induces inflammation in male reproductive system to cause sperm damages and subsequently infertility (Maresch et al., 2018). This phenomenon was found likely associated with re-polarization of tissue macrophages from an anti-inflammation M2-like subtype to a pro-inflammation M1-like subtype (Loveland et al., 2017), although the actual macrophage polarization is a complicated spectrum while M1 and M2 are just two extreme phenotypes (Loveland et al., 2017). However, the underlying mechanisms are not fully determined. Moreover, a practical approach to interfere with the progression of infertility is lacking.

A Jumonji-C (JmjC) domain is important for proteins to function as H3K27 demethylases, which catalyze the conversion of H3K27me3 (trimethylated) to H3K27me1 (monomethylated) in epigenetic control (Davis et al., 2021). Jumonji domain-containing protein D3 (JMJD3) has a tetratricopeptide repeat in addition to the JmjC domain and shares extensive homologous structures with other Jmjc proteins from inside and outside the JmjC domain. JMJD3 has been shown to induce M2-polarization in macrophages (Satoh et al., 2010).

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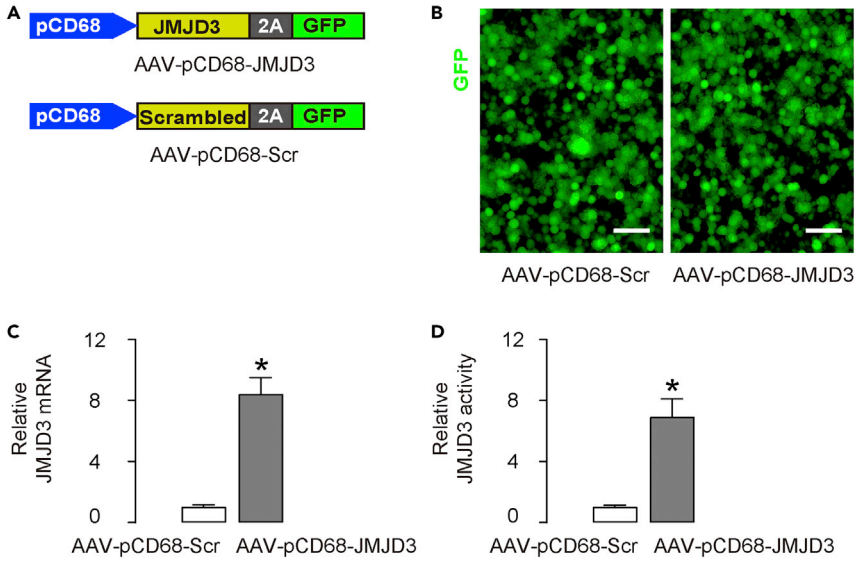

Figure 1. Generation of AAVs that increase JMJD3 specifically in macrophages

(A) AAVs carrying an M2-trigger, JMJD3, under a macrophage-specific CD68 promoter was generated. A scramble sequence was used as a control. GFP reporters were also included in the construct for assessment of the transduction efficiency.

(B) Cultured RAW264.7 cells successfully transduced with the viruses. GFP channel was shown.

(C) RT-qPCR for JMJD3.

(D) JMJD3 activity. *$p < 0.05$. N = 5. Scale bars are 50 μm.

In this study, we assessed the effects on the infertility of diabetic male mice through an *in vivo* approach that transduced the testicular macrophages back to the M2-like phenotype with adeno-associated viruses (AAV) carrying JMJD3 under a macrophage-specific CD68 promoter.

RESULTS

Generation of AAVs that increase JMJD3 specifically in macrophages

In this study, we aimed to induce M2-like macrophage polarization in the testis of the diabetic male mice, and to check whether this approach may have a therapeutic effect on diabetes associated with male infertility. Thus, we generated AAVs carrying an M2-trigger, JMJD3, under a macrophage-specific CD68 promoter. This virus allowed exclusive expression of JMJD3 in macrophages. A scramble sequence was used a control. GFP reporters were also included in the construct for assessment of the transduction efficiency (Figure 1A). We found that most of the RAW264.7 cells were successfully transduced with the viruses, evidence by the GFP expression (Figure 1B). Overexpression of JMJD3 in transduced RAW264.7 cells was confirmed by RT-qPCR (Figure 1C) and by JMJD3 activity (Figure 1D). Thus, these viruses were readily used for *in vivo* studies.

Orthotopic injection of AAVs in the testis does not alter diabetes

Next, we assigned male mice into four groups of five each. Group 1: mice only received an intraperitoneal injection of saline (control for STZ). Group 2: mice only received an intraperitoneal injection of STZ. Group 3: mice received an intraperitoneal injection of STZ and an orthotopic injection of AAVs carrying control Scr into the testis one week after STZ. Group 4: mice received an intraperitoneal injection of STZ and an orthotopic injection of AAVs carrying control JMJD3 into the testis one week after STZ (Figure 2A). Fasting blood glucose was monitored, showing that STZ-treated mice developed sustained hyperglycemia in 1 week, regardless later orthotopic injection of AAVs into the testis. Moreover, orthotopic injection of AAVs into the testis did not alter hyperglycemia (Figure 2B). Serum insulin was monitored, which similarly showed sustained reduction since one week after STZ, while orthotopic injection of AAVs into the testis did not alter serum insulin levels (Figure 2C). At sacrifice, the STZ-treated mice exhibited decreases in beta-cell mass to a similar degree, regardless of orthotopic injection of AAVs into the testis (Figures 2D and 2E). Together,

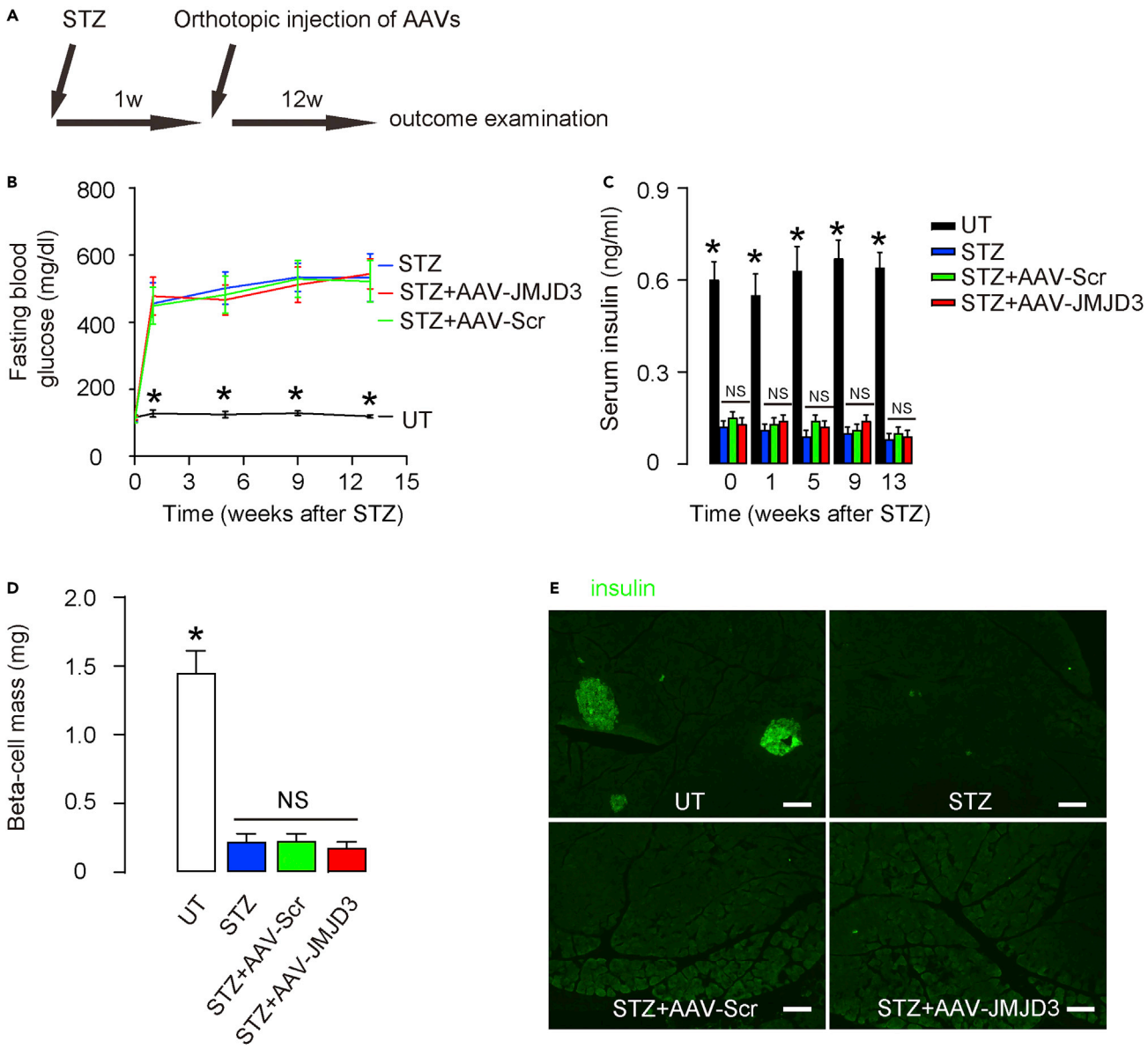

Figure 2. Orthotopic injection of AAVs in the testis does not alter diabetes

(A) Schematic of the study: we assigned male mice into four groups of five each. Group 1: mice only received an intraperitoneal injection of saline (control for STZ). Group 2: mice only received an intraperitoneal injection of STZ. Group 3: mice received an intraperitoneal injection of STZ and an orthotopic injection of AAVs carrying control Scr into the testis one week after STZ. Group 4: mice received an intraperitoneal injection of STZ and an orthotopic injection of AAVs carrying control JMJD3 into the testis one week after STZ. Mice were examined 12 weeks after treatment.

(B) Fasting blood glucose.

(C) Serum insulin.

(D) Beta-cell mass at sacrifice. (E) Representative immunofluorescent images for insulin in mouse pancreas. *$p < 0.05$. NS: non-significant. N = 5. Scale bars are 100 μm.

these data suggest successful establishment of chronic hyperglycemia in these STZ mice, and the diabetic status was not altered by orthotopic injection of AAVs into the testis.

JMJD3 expression in testicular macrophages improves mating capability of diabetic male mice

At the beginning of the 12 weeks after AAV injection (or the beginning of the 13 weeks after STZ injection; or one week before sacrifice), each male C57BL/6 mouse from four groups was caged with

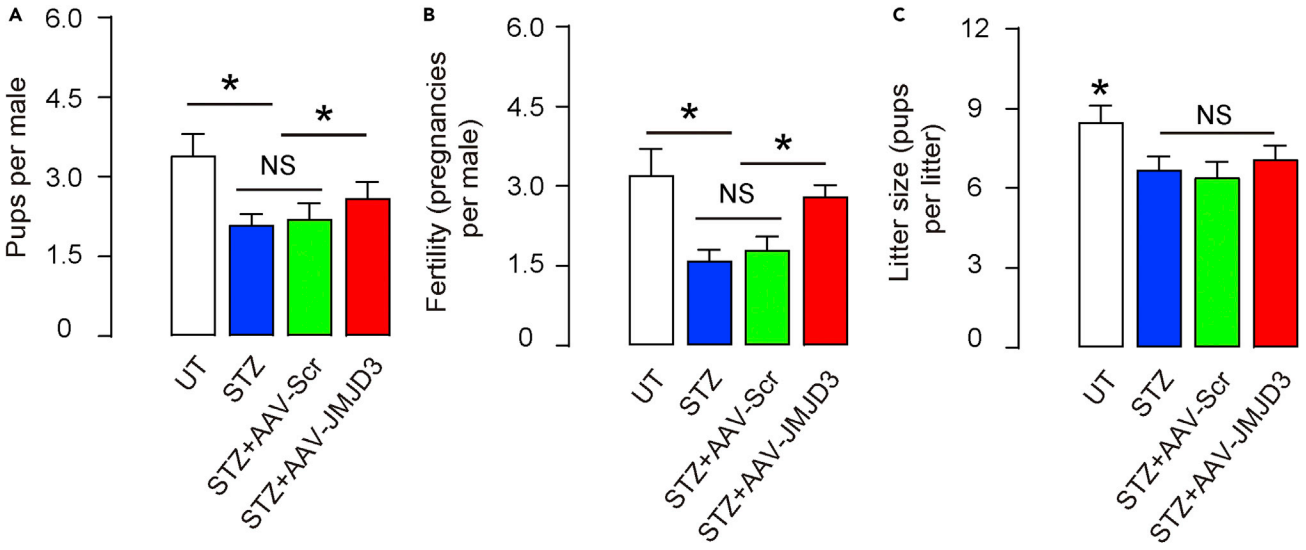

Figure 3. JMJD3 expression in testicular macrophages improves mating capability of diabetic male mice

At the beginning of the 12 weeks after AAV injection (or the beginning of the 13 weeks after STZ injection; or one week before sacrifice), each male C57BL/6 mouse from four groups was caged with four age-matched untreated female C57BL/6 mice for one week before they were removed from the females for final analysis followed by sacrifice.

(A) Vaginal mucous pups in females.

(B) Pregnancies per male.

(C) Litter sizes for successful pregnancies. *$p < 0.05$. NS: non-significant. N = 5.

four age-matched untreated female C57BL/6 mice for one week before they were removed from the females for final analysis followed by sacrifice. The females were monitored for vaginal mucous plugs, pregnancies, and litter sizes for successful pregnancies. By a qualitative observation, we did not detect any difference in the mating desire of the male mice regardless of their diabetic status. However, we found that the plug number from all females was significantly reduced when they were caged with diabetic male. The plug number did not increase when male diabetic mice received orthotopic injection of control Scr-AAVs, but significantly increased when male diabetic mice received orthotopic injection of JMJD3-AAVs (Figure 3A). Moreover, the total female pregnancies for one male mouse were also reduced when the diabetic male mice were used, while orthotopic injection of JMJD3-AAVs but not control Scr-AAVs significantly increased total female pregnancies (Figure 3B). Furthermore, the pups per litter in the successful pregnant female mice were quantified, without showing improvement with orthotopic injection of JMJD3-AAVs (Figure 3C). Together, these data suggest that JMJD3 expression in testicular macrophages improves mating capability of diabetic male mice.

JMJD3 expression in testicular macrophages improves sperm motility

Next, we performed computer-assisted sperm motility analysis. We found that the motile percentage of sperms was significantly reduced in diabetic male, while orthotopic injection of JMJD3-AAVs but not control Scr-AAVs significantly increased the motile percentage of sperms (Figure 4A). Similar pattern was detected in curvilinear velocity (Figure 4B) but not in straight line velocity (Figure 4C). Thus, JMJD3 expression in testicular macrophages improves sperm motility.

JMJD3 expression in testicular macrophages increases testosterone

Alterations in some sex-associated hormones have been detected in infertile male. Thus, we examined the levels of luteinizing (LH), testosterone, and follicle-stimulating hormones (FSH) in serum and the levels of testosterone in testis. While no difference was detected for LH and FSH among four groups (Figures 5A and 5B), significant reduction in testosterone was detected in both serum and testis in diabetic male (Figures 5C and 5D). The reduction in serum and testicular testosterone was significantly attenuated by orthotopic injection of JMJD3-AAVs but not by orthotopic injection of control Scr-AAVs (Figures 5C and 5D). Hence, JMJD3 expression in testicular macrophages increases testosterone.

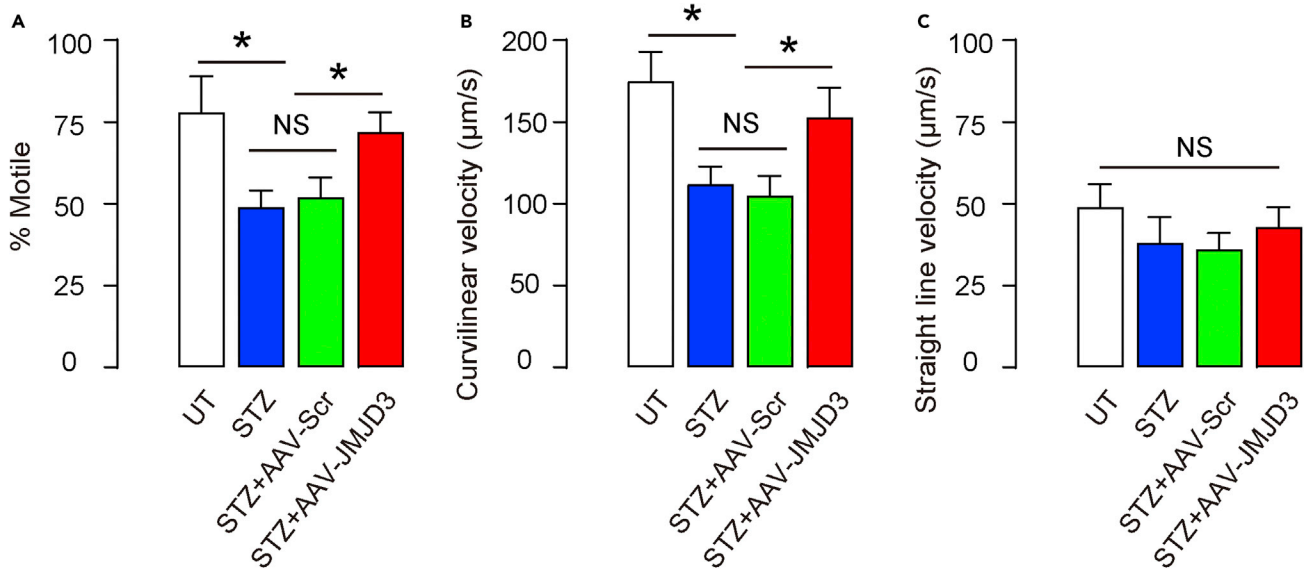

Figure 4. JMJD3 expression in testicular macrophages improves sperm motility

Computer-assisted sperm motility analysis was performed.

(A) the motile percentage of sperms.

(B and C) curvilinear velocity (C) straight line velocity (C). *$p < 0.05$. NS, non-significant. N = 5.

JMJD3 repolarizes testicular macrophages back to M2-polarized phenotype

Finally, we validated the effects of JMJD3 on macrophage polarization. First, the digested testicle cells were subjected to CD68 and GFP double FACS. GFP was exclusively detected in CD68 $^{+}$ cell fraction, confirming the specificity of the CD68 promoter (Figure 6A). GFP signals were exclusively detected in the testis that had received viral injection, but not from blood, liver, lung, and kidney in the mice that had received virus or from mice that had not received viral injection (Figure 6B). Next, testicular macrophages were isolated and subjected to flow cytometry for CD68 (total macrophages) and CD163 (a specific marker for M2 macrophages). We found that diabetes induced an M1-polarization of testicular macrophages, which was reversed by JMJD3 (Figures 6C and 6D). The alterations in testicular macrophage polarization were confirmed by gene expression of some M2 markers (CD206 and Arginase 1 (ARG1)) and M1 markers (iNOS, IL-6, and TNFα) (Figure 6E). Because only one injection of AAVs was applied, the resident testicular macrophages rather than macrophages derived from circulation appeared to be the major player in this model. Together, our data suggest that oriented JMJD3-therapy on macrophages may be a promising strategy to treat diabetes-associated male infertility.

DISCUSSION

Normally, testicular macrophages are immune cells that have an important defensive role in protecting sperm (Pollanen and Maddocks, 1988). However, the role of macrophages in sperm protection is also very complicated (Loveland et al., 2017). Macrophages not only migrate to sites of infection and phagocytose pathogens to prevent them from getting into the male reproductive system but also have a critical immune-modulative function to coordinate the proper organ function and regeneration after injury (Wang et al., 2021). Although testicular macrophages are known to derive from both embryogenesis and bone marrow stem cells in adults, here our data suggest that under a diabetic status, the early committed testicular macrophages from embryo may be more important, since one single injection of AAVs showed a significant effect on macrophage polarization.

Many different mechanisms have been shown as the cause of male infertility by diabetes or chronic hyperglycemia. Among these possible causes, macrophage polarization has been regarded as a co-occurring event, rather than a key regulator (Olive et al., 1985; Pelliccione et al., 2009; Yu et al., 2014). In our study, we showed intervention of testicular macrophage polarization nearly abolished the effects of diabetes on male infertility, raising the hypothesis that pro-inflammatory macrophage polarization may be a key factor to lead to male infertility under a diabetic status.

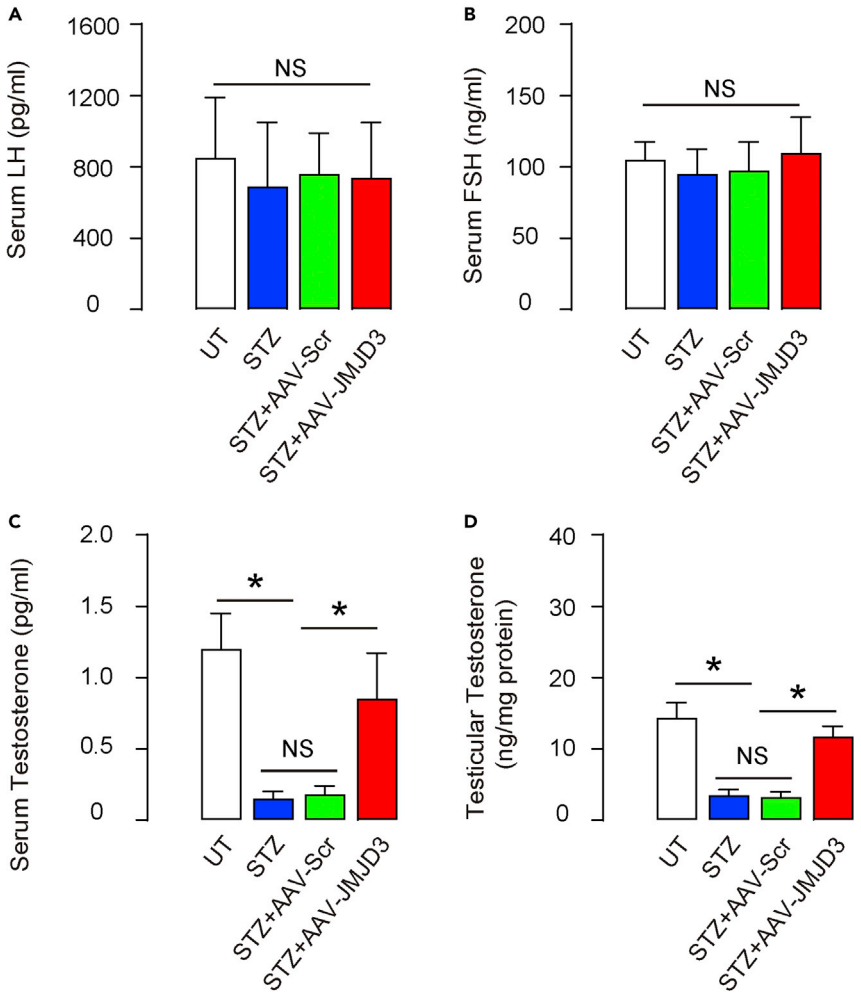

Figure 5. JMJD3 expression in testicular macrophages increases testosterone

(A–D) The levels of LH (A), FSH (B), and testosterone (C) in serum and the levels of testosterone in testis (D). *p < 0.05. NS: non-significant. N = 5.

Recently, the role of resident macrophages has been shown to play critical roles in different diabetes (Carrero et al., 2017; Liu et al., 2012; Meshkani and Vakili, 2016; Thornley et al., 2016; Yang et al., 2020; Zhu et al., 2021). Here, our study demonstrates the pivotal role of macrophage polarization in regulation of male infertility in diabetes. The diabetes-induced M1-polarization of testicular macrophages could result from an epigenetic alteration in diabetic status (Fu et al., 2014; Knipper et al., 2019). A systemic intervention of resident macrophages rather than a local treatment may be more attractive for a combined therapy for multiple diabetes-associated diseases, which warrants further investigations.

Limitations of the study

In this study, we did not examine the phenotypic changes in macrophages from different origin. Because interstitial macrophages in the testis are of embryonic origin, while circulation-derived testicular macrophages are mainly peritubular, it may be interesting to address one population using a population-specific promoter to drive JMJD3 in the future study.

ETHICAL APPROVAL

All the experiments here have been approved by the research committee at Shanghai Ruijin Hospital, Shanghai Jiao Tong University School of Medicine. All animal experiments were approved by the

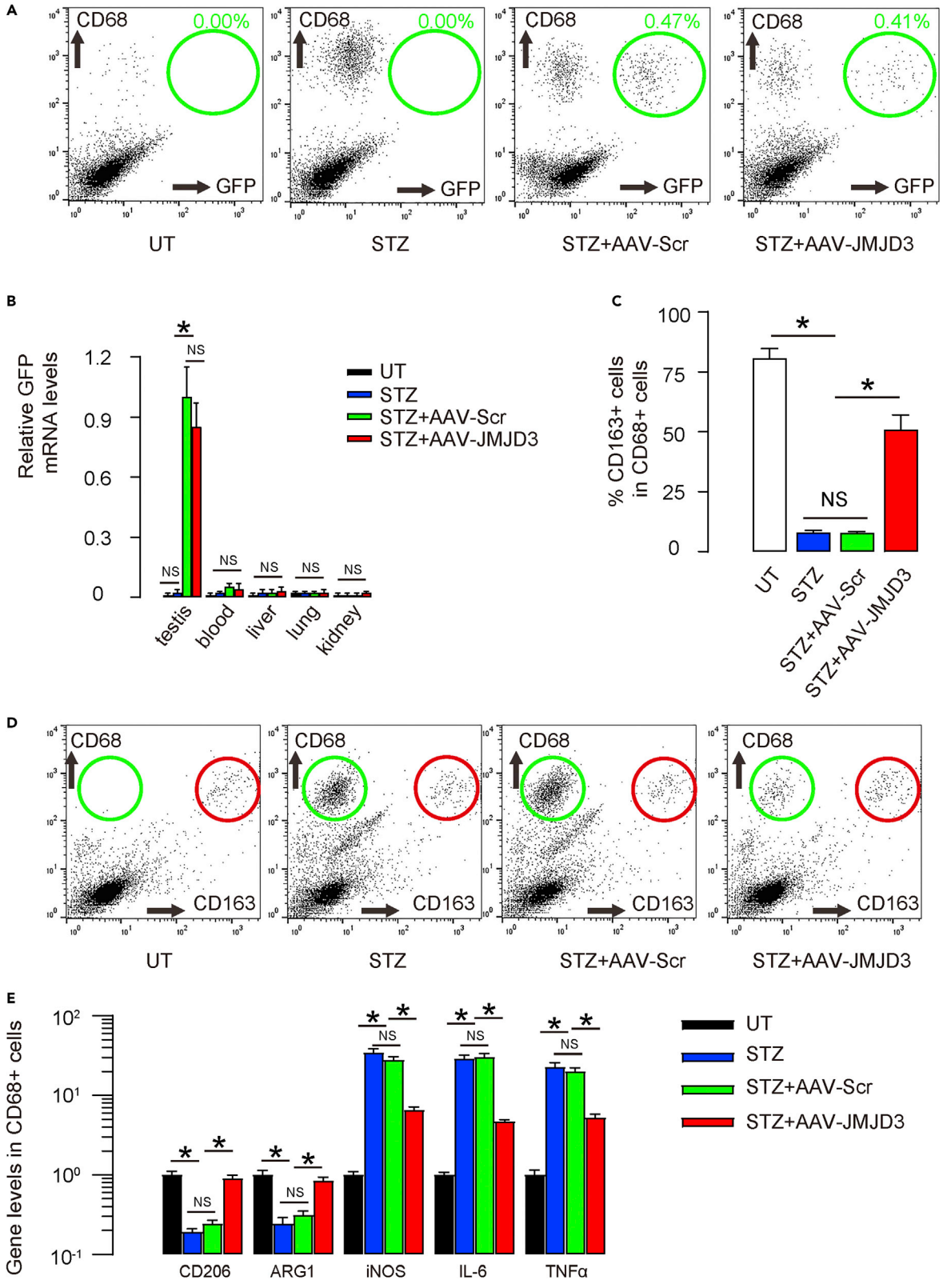
A
CD68
0.00%
GFP
UT
STZ
0.47%
STZ+AAV-Scr
0.41%
STZ+AAV-JMJD3
B
Relative GFP mRNA levels
UT
STZ
STZ+AAV-Scr
STZ+AAV-JMJD3
testis
blood
liver
lung
kidney
NS
C
% CD163+ cells in CD68+ cells
D
CD163
E
Gene levels in CD68+ cells
CD206
ARG1
iNOS
IL-6
TNFα

Figure 6. JMJD3 repolarizes testicular macrophages back to M2-polarized phenotype

(A) The digested testicle cells were subjected to CD68 and GFP double FACS, showing that GFP was exclusively detected in CD68 $^{+}$ cell fraction.

(B) RT-qPCR for GFP in testis, blood, liver, lung, and kidney in mice.

(C and D) Testicular macrophages were isolated and subjected to flow cytometry. (C) Quantification for % CD163 + cells in total CD68 $^{+}$ cells. (D) Representative flow charts for CD68 and CD163.

(E) RT-qPCR for M2 markers (CD206 and Arginase 1 (ARG1)) and M1 markers (iNOS, IL-6, and TNFα) in testicular CD68 $^{+}$ macrophages. *p < 0.05. NS, non-significant. N = 5.

Institutional Animal Care and Use Committee at Shanghai Ruijin Hospital, Shanghai Jiao Tong University School of Medicine (Animal Welfare Assurance).

STAR★METHODS

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AUTHOR CONTRIBUTIONS

XZ was responsible for study design and administrative support. All authors contributed to data collection and analysis, manuscript writing, and final approval of manuscript submission.

DECLARATION OF INTERESTS

The authors have declared that no competing interests exist.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
BV421-conjugated anti-CD68	Becton-Dickinson Biosciences	566388
Alexa Fluor 647-conjugated anti-CD163	Becton-Dickinson Biosciences	562669
Chemicals, peptides and recombinant proteins		
Streptozotocin	MilliporeSigma	S0130
Critical commercial assays		
JMJD3 chemiluminescent Assay Kit	BPS Bioscience	#50406
Omniscript reverse transcription kit	Qiagen	#205113
Experimental models: Cell lines		
RAW 264.7	American Type Culture Collection	TIB-71
Experimental models: Organisms/strains		
C57BL/6	SLAC	C57BL/6Slac
Oligonucleotides		
CD68 promoter plasmid	Addgene	#34837
Software and algorithms		
Flowjo	Flowjo LLC	version 10

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the corresponding author, Dr. Aijun Zhang (cliff26@126.com).

Materials availability

This study did not generate new unique reagents. Primers used are all purchased from Qiagen.

Data and code availability

Data reported in this paper will be shared by the lead contact upon reasonable request. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon reasonable request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Four groups of mice (5 mice each) were used in this study. Group 1: mice only received an intraperitoneal injection of saline (control for streptozotocin (STZ, MilliporeSigma, Burlington, MA 01803, USA)). Group 2: mice only received an intraperitoneal injection of STZ. Group 3: mice received an intraperitoneal injection of STZ and an orthotopic injection of AAVs carrying control Scr into the testis one week after STZ. Group 4: mice received an intraperitoneal injection of STZ and an orthotopic injection of AAVs carrying control JMJD3 into the testis one week after STZ.

METHOD DETAILS

Cell culture for mouse macrophage cell line

RAW 264.7 is a mouse M1-like macrophage cell line. RAW 264.7 cells were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA), and cultured in RPMI 1640 Medium (MilliporeSigma) in a humidified chamber with 5% CO_2 at 37°C.

Generation of AAVs to transduce macrophages

Mouse open reading frames for JMJD3 were amplified from RAW 264.7 cells. CD68 promoter was obtained from Addgene (#34837, Watertown, MA, USA) provided by dr. Peter Murray (Lang et al., 2002). Both constructs were ligated into a backbone plasmid pAAV-cmv-GFP. Transfection of cells was performed using a Lipofectamine 3000 reagent (Thermofisher, CA, Carlsbad, USA). The serotype of AAV used AAV2. The prepared virus (JMJD3 or control scrambled sequence (Scr)) was stored at -80°C. A dot-blot assay was used to determine the titration of viruses.

Diabetes

An intraperitoneal injection of STZ at 120 mg/kg body weight was chosen to induce diabetes in 12-week-old male C57BL/6 mice (SLAC Laboratory Animal, Shanghai, China) by. This modest dose allows development of hyperglycemia but relatively long survival of the mice. One week later, the development of diabetes in mice was confirmed by fasting blood glucose of the mice reaching no less than 350 mg/dl. The diabetic mice received an orthotopic injection of 5 μl 10^{11} AAVs carrying JMJD3 or control Scr into the testis. Fasting blood glucose has been described before, serum insulin and beta-cell mass measurement followed the protocol as described (Garofano et al., 1998).

Mating assays

At the beginning of the 12 weeks after AAV injection (or the beginning of the 13 weeks after STZ injection; or one week before sacrifice), each male C57BL/6 mouse from 4 groups was caged with 4 age-matched untreated female C57BL/6 mice. All the mice were provided with standard pelleted chow. The male was isolated from the females after one week for final analysis before sacrifice. Vaginal mucous plugs were assessed in females. Pregnancies and litter sizes for successful pregnancies were also determined.

Sperm motility

Motile percentage, curvilinear velocity and straight-line velocity were assessed in a computer-assisted sperm motility analysis.

Endocrinology

Luteinizing (LH), Testosterone, and follicle-stimulating hormones (FSH) levels were analyzed as described (van der Spoel et al., 2002).

Quantitative real-time PCR (RT-qPCR) and JMJD3 chemiluminescent assay

Total RNA was extracted with RNeasy mini kit (Qiagen, Hilden, Germany). An Omniscript reverse transcription kit (Qiagen) was used to generate complementary DNA (cDNA) from 2 μg of total RNA. A Quantitect SyBr green PCR system (Qiagen) was applied for RT-qPCR using Qiagen-commercial primers. A 2-△△Ct method was used for data analysis, in which the standardization of the values of genes was obtained through sequentially normalization against Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and the experimental control. JMJD3 activities were determined with a JMJD3 chemiluminescent Assay Kit (BPS Bioscience, Inc., San Diego, CA, USA).

Sperm motility analysis

Spermatozoa were collected from vasa deferentia and caudae epididymides and put in M2 medium (Sigma-Aldrich, Los Angeles, CA, USA) at 37°C for 1 h. A Hobson Sperm Tracker (Hobson Vision, Baslow, Derbyshire, U.K.) was used for a computer-assisted sperm motility analysis.

Flow cytometry

Mouse testis was dissected out and digested in serum-free RPMI 1640 Medium supplemented with 0.5% collagenase (Sigma-Aldrich) for 30 min followed by combined of 0.5 mg/ml trypsin (Sigma-Aldrich) and 1ug/ml DNase (Sigma-Aldrich) for 15 min. The obtained digests that passed a 66 nm filter were subjected to flow cytometry analysis and sorting. For flow cytometry, the antibodies used BV421-conjugated anti-CD68 and Alexa Fluor 647-conjugated anti-CD163 (Becton-Dickinson Biosciences). GFP was detected by direct fluorescence. The dead cells and doublets were excluded based on forward and side scatter displays in the FACS. The gating for certain fluorescent channels was decided based on the analysis of the isotype control of the applied antibodies. Since the populations are clear, no compensation was used during

gating and analysis. The total macrophages were determined by a total gate for CD68. The M2 macrophages were determined by a gate for both CD68 and CD163 positivity. The flow cytometry was performed with a BD FACSAria™ II Cell Sorter. A Flowjo (version 10, Flowjo LLC, Ashland, OR, USA) was used to analyze the experimental data.

QUANTIFICATION AND STATISTICAL ANALYSIS

Mean ± standard deviation (SD) was used to present experimental data after analyzed with a one-way analysis of variance (ANOVA) test followed by the Fisher's Exact Test (GraphPad Software, Inc. La Jolla, CA, USA). A value of $p < 0.05$ was considered statistically significant.