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# Sialylation Facilitates the Maturation of Mammalian Sperm and Affects Its Survival in Female Uterus<sup>1</sup>

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## ABSTRACT

Establishment of adequate levels of sialylation is crucial for sperm survival and function after insemination; however, the mechanism for the addition of the sperm sialome has not been identified. Here, we report evidence for several different mechanisms that contribute to the establishment of the mature sperm sialome. Directly quantifying the source of the nucleotide sugar CMP-beta-N-acetylneuraminic acid in epididymal fluid indicates that transsialylation occurs in the upper epididymis. Western blots for the low-molecular-mass sialoglycoprotein (around 20–50 kDa) in *C57BL/6* mice epididymal fluid reflect that additional sialome could be obtained by glycosylphosphatidylinositol-anchored sialoglycopeptide incorporation during epididymal transit in the caput of the epididymis. Additionally, we found that in *Cmah* (CMP-N-acetylneuraminic acid hydroxylase)–/– transgenic mice, epididymal sperm obtained sialylated-CD52 from seminal vesicle fluid (SVF). Finally, we used *Gfp* (green fluorescent protein)+/+ mouse sperm to test the role of sialylation on sperm for protection from female leukocyte attack. There is very low phagocytosis of the epididymal sperm when compared to that of sperm coincubated with SVF. Treating sperm with *Arthrobacter ureafaciens* sialidase (AUS)

increased phagocytosis even further. Our results highlight the different mechanisms of increasing sialylation, which lead to the formation of the mature sperm sialome, as well as reveal the sialome's function in sperm survival within the female genital tract.

*phagocytosis, sialic acid, sperm*

## INTRODUCTION

All cell surfaces in nature are covered by a glycocalyx, a dense “sugar coat” formed by a complex array of the oligo- and polysaccharides attached to glycoproteins and glycolipids [1]. Sperm are coated with a thick (~70 nm) glycocalyx rich in sialic acids (Sias) [2]. Sias are nine-carbon-backbone amino sugars abundantly found on mammalian cell surfaces and secretions [3]. These acidic sugars are important in a large number of biological functions ranging from either structure or function roles based on their negative charge to involvement in molecular recognition during development, immune regulation, and carcinogenesis [4]. There are over 50 different forms of Sias in nature, but the two most common Sias found in mammals are *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc). Humans only express Neu5Ac as an ancient inactivating mutation of the *Cmah* gene and have abolished their ability to modify CMP-Neu5Ac into CMP-Neu5Gc [5]. The common linkage types of Sias to the underlying glycan chains are Sia  $\alpha$ 2-3 and Sia  $\alpha$ 2-6 [6]. A third linkage type,  $\alpha$ 2-8, is found, e.g., in cells of the central nervous system and sperm [7–9].

As with other mammalian cells, Sias are found as the outermost monosaccharide, capping the majority of glycans at the sperm cell surface. The sperm sialome refers to the total Sias present in and on sperm. We have previously quantified Sias on mature sperm cells and found that each sperm bears tens of millions of Sia molecules [10]. The sperm sialome is established during spermatogenesis, during epididymal maturation, and by incorporation of sialylated seminal fluid components into the sperm membrane during ejaculation [11, 12]. As allogeneic cells, sperm face important challenges once introduced into the female reproductive tract [13]. In fact, female immune factors both humoral and cellular lead to the rapid demise of the vast majority of sperm. The leukocytic

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reaction unleashes large numbers of leukocytes in the uterus shortly upon copulation [14, 15]. Antibodies and complements are also components of female reproductive tract secretions [16], and highly sialylated epididymal glycoproteins such as beta defensins have been shown to mask sperm antigens to antibody binding [17]. The complement system also relies in part on appropriate sialylation of cells to detect self and inhibit activation based on factor H binding [18], and as such, appropriate sialylation could be as important for sperm survival as the presence of multiple-complement down-regulating molecules such as CD55 and CD59 [19]. Insufficient decoration with Sias is likely to put sperm at increased risk of female immune intolerance.

Sia content of mammalian sperm has been shown to correlate positively with protection from phagocytosis but negatively with the capacity of sperm to bind to zona pellucida of the ovum [20, 21]. It appears that Sia decoration of sperm is subject to temporal modulation during sperm maturation. Existing studies of Sia modulation in sperm have mostly relied on indirect measures of Sia gain, including transferase activity and ganglioside content [12, 22]. We here profiled the sialome of sperm from spermatogenesis in the testis, in transition through the epididymal lumen and maturation, and finally while in contact with seminal fluid during ejaculation. Also, we investigated the mechanisms responsible for the addition of the sialome during sperm maturation, and revisited the role of sperm sialylation for protection after insemination.

## MATERIALS AND METHODS

### Animal Care and Use

The research was conducted using *C57BL/6* mice, *Cmah*<sup>-/-</sup> mice, and *Gfp* (green fluorescent protein)<sup>+/+</sup> mice following the approval of the University of California (UC) San Diego Animal Care and Use Committee. Animals used in these studies were maintained under UC San Diego IACUC Protocol #S01227 and euthanized according to the principles and procedures described in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

### Sperm Collection and Preparation of Sperm Extracts

Sperm were harvested from the cauda epididymis of euthanized 12- to 20-wk-old males. Cauda epididymis was crushed and kept on a shaker at room temperature (RT) for 10 min, then centrifuged for 30 sec at 500 × *g*. Sperm were washed with Biggers-Whitten-Whittingham medium (BWW; pH 7.5; 91.06 mM NaCl, 4.78 mM KCl, 1.71 mM CaCl<sub>2</sub>, 1.71 mM KH<sub>2</sub>PO<sub>4</sub>, 2.44 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 5.55 mM glucose, 0.25 mM sodium pyruvate, 21 mM HEPES at pH 7.4, 25 mM NaHCO<sub>3</sub>, 21.5 mM sodium lactate) by centrifugation at 800 × *g* for 1 min and subjected to a swim-up procedure in 500 μl BWW at 37°C and 5% CO<sub>2</sub> for 30 min following published protocols [23]. The same procedure was used to isolate sperm from the caput and the cauda. Mouse membrane sperm proteins were extracted following published protocols [24].

### Collection of Mouse Epididymal Fluid

Fluids with sperm from different epididymal regions were collected. The samples were gently washed three times with BWW. The supernatant was pooled, concentrated, and then centrifuged to remove the pellet. Protein was quantified and kept at -20°C until used for assays.

### In Vitro Seminal Vesicle Fluid Incubation with Sperm

Seminal vesicles were isolated from blood vessels and accessory glands by careful dissection. The gland fluid contents were manually expressed into microcentrifuge tubes. Twenty microliters of seminal fluid was then mixed with 100 μl of physiological BWW and placed in a humidity chamber at 37°C for 10 min. The suspension was centrifuged at 1000 × *g* for 5 min and the supernatant was collected for sperm incubation; 10<sup>6</sup> sperm were added to 50 μl diluted seminal vesicle fluid (SVF) and incubated at 37°C and 5% CO<sub>2</sub> for 15 min [25].

### Retrieving Leukocytes of Female Mice 15 h after Mating and Coincubation with Sperm

A single male (*C57BL/6*) was caged with two females in estrus stage overnight. The following morning the females were examined for the presence of vaginal plugs as an indication of successful mating. Females were euthanized 15 h after mating, the whole uterus was excised, the two horns were separated, and each horn was clamped shut with forceps. The uterine horns were flushed with 200 μl PBS (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>) using a 28-gauge syringe. Live sperm were removed by swim-up method to isolate leukocytes. The leukocytes were then coincubated with the *Gfp*<sup>+/+</sup> sperm group at 37°C and 5% CO<sub>2</sub> for 4 h.

### Western Blot

Mouse membrane sperm proteins were extracted following the protocol of Shetty et al. [24]. Mouse SVF proteins were separated by loading SVF directly on 10% SDS-PAGE (Bio Rad). Proteins were either transferred to PVDF membrane (Bio Rad) or stained with Coomassie stain (Bio Rad). For detection of α2-3 and α2-6 sialoglycoprotein, the membranes were blocked with 1% bovine serum albumin (BSA) in Tris-buffered saline with Tween (TBST; 0.1%) and incubated overnight at 4°C. After washing, the blots were incubated for 30 min with 1:6000 biotinylated *Sambucus nigra* lectin (SNA; Vector Laboratories) and *Maackia amurensis* lectin 2 (MAL-II; Vector Laboratories) in 1% BSA TBST, washed, then incubated with horseradish peroxidase (HRP)-conjugated streptavidin (Vector Laboratories) at concentration of 1:100 000 for 1 h at RT. Meanwhile, mild NaIO<sub>4</sub> treatment was used as control (membrane were washed with H<sub>2</sub>O and incubated with 20 mM NaIO<sub>4</sub> in PBS [pH 6.5] at RT 30 min in the dark). For Neu5Gc staining, blots were incubated for 1 h at RT in 1:50 000 affinity purified chicken IgY (Sialix, Inc.) in TBST, 0.5% fish gelatin (Sigma-Aldrich), and 2 μg/ml of purified BSA (double blocking). Blots were washed and incubated with secondary HRP-conjugated donkey anti-chicken antibody (1:5000; Biologend) for 1 h at RT. Blots were developed using Pierce luminescent substrate (Thermo Scientific).

### Analysis of Flow Cytometry and Immunofluorescence

To assess lectin binding, sperm were fixed with 3% freshly thawed paraformaldehyde (PFA) for 20 min at RT, washed with PBS, and then blocked with 1% BSA TBST. Biotinylated lectins of SNA and MAL-II were used at a concentration of 1:1000 at RT for 30 min and detected with streptavidin conjugated with Alexa Fluor 555/488 secondary antibodies (Biologend) at a concentration of 1:1000 at RT for 30 min. To evaluate CD52 expression, spermatozoa were fixed with freshly thawed 3% PFA for 20 min at RT, washed with PBS, and then blocked with 1% BSA PBS. Anti-mouse CD52 antibody (Biologend) was used at 1:400 at 4°C for 1 h, and secondary donkey anti-mouse antibody conjugated with Alexa Fluor 647 (Biologend) was used at 1:500 at 4°C for 1 h. For APC/CY7-F4/80, the mixed cells after coincubation were washed with PBS, then incubated with the antibody at 1:500. Stained sperm were analyzed by flow cytometry on a BD FACScalibur. A smear of stained sperm was counterstained with 4',6-diamidino-2-phenylindole (DAPI; 1:500 000; Sigma-Aldrich), and also made for microscopy and microphotography on an applied precision Deltavision inverted deconvolution system (Zeiss).

### Immunohistochemistry

The male genital tissues including testis and epididymis were harvested, then frozen by surrounding with Optimum Cutting Temperature compound (Vector Lab) and immersed in a bath of dry ice and 2 methyl butane slush. Five-micrometer sections were prepared by sectioning on a cryostat microtome. Sections were fixed with acetone for 10 min, blocked with 0.03% H<sub>2</sub>O<sub>2</sub> for 30 min, and then blocked with 1% BSA for 10 min at RT. SNA, MAL-II, and anti-Neu5Gc antibody (Sialix, Inc.) were used at a concentration of 1:500 for 1 h at RT, and then HRP-anti-streptavidin and HRP-anti-chicken antibody were used at 1:500 for 30 min at RT. Sections were developed with 3-amino-9-ethylcarbazole (AEC) substrate chromogen (Vector Laboratories) and counterstained with hematoxylin (Sigma-Aldrich). Microphotographs were taken under a Zeiss fluorescent microscope.

### Determination of CMP-Sia by High-Performance Anion-Exchange Chromatography-Pulsed Amperometric Detector

The regional epididymal fluids were detected with high-performance anion-exchange chromatography-pulsed amperometric detector (HPAEC-PAD; Dionex ICS 3000 system). Elutions were carried out at 1 ml/min with the

analytical column Carbo Pac PA 1. The solvents were water (E1), 0.1 M sodium hydroxide (E2), and 1 M sodium acetate in 0.1 M sodium hydroxide (E3). The samples were injected into the column and elution was performed by the following gradient:  $T_0 = (E1:E2:E3::94.5:5.0:0.5)$ ;  $T_{50} = (E1:E2:E3::45:10:45)$ .

### Arthrobacter ureafaciens Sialidase Treatment

*Arthrobacter ureafaciens* sialidase (AUS; EY Laboratories) was used at 15 mU/100 000 sperm cells for partial desialylation. The treatment time was 20 min.

### Statistical Analysis

Data sets were analyzed for statistical significance using a one-way ANOVA test. All values shown are means  $\pm$  SEM.  $P \leq 0.05$ ,  $P \leq 0.01$ , and  $P \leq 0.001$  were considered statistically significant versus control.

## RESULTS

### Sialome Profile of Mouse Sperm at Different Stages of Development

There is some information about the sperm sialome in some mammalian species [26]. To understand the sialome on sperm during the maturation process, we determined the sialome of mouse sperm using lectin and immunohistochemical methods. We stained mouse testes and epididymides using SNA (specific for Sia  $\alpha$ 2-6), MAL-II (specific for with Sia  $\alpha$ 2-3), and anti-Neu5Gc antibody (polyclonal IgY raised in chicken against Neu5Gc). We found no Sias expressed on testicular sperm during early developmental stages except for Sia  $\alpha$ 2-3, which was strongly expressed on spermatocytes. This indicates that the sialome first appears on spermatocytes as Sia  $\alpha$ 2-3 of Neu5Ac. Mature mouse sperm expressed more than 20% of their Sias as Neu5Gc. The histochemistry in Figure 1B indicates that most of the Neu5Gc appears on sperm only after it leaves the testes. Once sperm were outside the testes, there was an incremental increase of Sias on sperm from caput to caudal region of the epididymis, including  $\alpha$ 2-3 and  $\alpha$ 2-6 Sias (Fig. 1, A and B). There was also abundant Sia on the adjacent epithelia of the epididymal duct (Fig. 1B), from which certain sialoglycoconjugates might be acquired by the membrane of maturing sperm. In order to explore the acquisition of the sperm sialome, we used fluorescent lectin staining followed by quantification using flow cytometry. Analysis of sialylation levels of epididymal sperm from proximal to distal regions (caput to cauda) provided evidence for an incremental increase in Sia coating of the mouse sperm surface (Fig. 1C).

### Neu5Ac Is Transferred to Sperm from Luminal CMP-Neu5Ac During Epididymal Transit

It has been previously reported that there are different concentrations of sialyltransferase in the lumen of rat epididymis from caput and cauda [27]. The coincubation of exogenous CMP-Neu5Ac and sialyltransferase with mouse sperm showed that Sia can be added to the sperm surface in vitro from exogenous CMP-Neu5Ac [12]. These findings are provocative, as CMP-Neu5Ac is not usually found outside of cells. To identify the presence of CMP-Neu5Ac in epididymal fluid, which is an absolute prerequisite for extracellular sialyltransferase function, we collected epididymal fluid from different regions of the epididymis (Fig. 2A) separated into caput 1, caput 2, corpus, and cauda. We performed determination and quantification of extracellular CMP-Sia by HPAEC-PAD using a standard CMP-Neu5Ac positive control. A blank control and female mouse uterine fluid were set as

negative and positive control respectively. A small amount of CMP-Neu5Ac, but no CMP-Neu5Gc, was detected in wild-type (WT) mice. The concentration was highest in caput 2 and corpus compared with testis, caput 1, and cauda (Fig. 2, B and C), suggesting that CMP-Sia is transferred to sperm by sialyltransferase mainly in epididymal caput and corpus during transition.

### Glycosylphosphatidylinositol-Anchored Sialoglycoproteins Are Obtained by Sperm During Epididymal Transition

Known mechanisms for the gain of sialoglycoconjugates by cells include the incorporation of sialylated glycosylphosphatidylinositol (GPI)-anchored glycopeptides or glycoproteins and the incorporation of gangliosides (sialylated glycosphingolipids) [22]. A further possibility is the recently demonstrated coating of maturing sperm by a beta defensin (Bin1b in rat, Defb22 in mouse, and DEFB126 in primates) with a typical cationic domain and a tail domain that contains numerous sialylated *O*-glycans [28]. The configuration of the mature sperm sialome thus likely reflects epididymal sialylation as well as incorporation of GPI-anchored sialoglycopeptides, gangliosides, and sialylated defensins from epididymal fluid. To test the degree of acquisition of GPI-anchored sialoglycoproteins during epididymal transit, we isolated epididymal fluid and used lectin binding and high-affinity anti-Neu5Gc antibody staining of sialoglycoprotein separated by reduced SDS-PAGE. Because this procedure is very sensitive and less specific, we used Neu5Ac  $\alpha$ 2-3/6-human sera albumin (HSA) as positive control. We found sialoglycoproteins throughout the epididymis, but the amount and composition varied between fluids from different segments of the epididymis (Fig. 3A). Glycoproteins with  $\alpha$ 2-3 Sia were mainly expressed in caput 2 as low molecular mass (around 20–50 kDa), whereas glycoproteins with  $\alpha$ 2-6 Sia were mainly expressed in caput 1 as low molecular mass (around 20–50 kDa). Staining of Western blots for the Sia Neu5Gc in WT mice epididymal fluid revealed abundant presence of glycoproteins decorated with this Sia throughout (Fig. 3, B–D). Thus, the expression of low-molecular-mass sialoglycoprotein indicates that additional sialome could be obtained by GPI-anchored sialoglycopeptide incorporation during epididymal transit, and it appears more likely that this transfer occurs in the caput rather than in the corpus and cauda of epididymis.

### Sialome Gain from SVF During In Vitro Incubation

Prior to their release by ejaculation, sperm are stored under physiological stimulation in the epididymis for the final maturation processes. The contact with seminal plasma provides an additional opportunity for the modulation of the sperm sialome, as sperm can assimilate gangliosides and GPI-anchored sialoglycolipids from seminal fluid [22]. The quantification of gangliosides requires large amounts of glycolipid, making it difficult to measure the total gangliosides that sperm acquire from seminal fluid. To investigate the mechanism of the addition of GPI-anchored sialoglycoprotein, we focused on the GPI-anchored sialoglycopeptide CD52, a highly sialylated glycopeptide with sperm-specific glycoforms, which has been demonstrated to be obtained by mouse sperm only after ejaculation [29]. In contrast, CD52 is expressed in the epididymis in humans. We took advantage of the *Cmah*<sup>-/-</sup> transgenic mouse, which lacks the Sia Neu5Gc and has only Neu5Ac [5]. We performed in vitro incubation of epididymal sperm and SVF following the reported procedure [30] but used sperm from *Cmah*<sup>-/-</sup> mice coincubated with SVF from WT

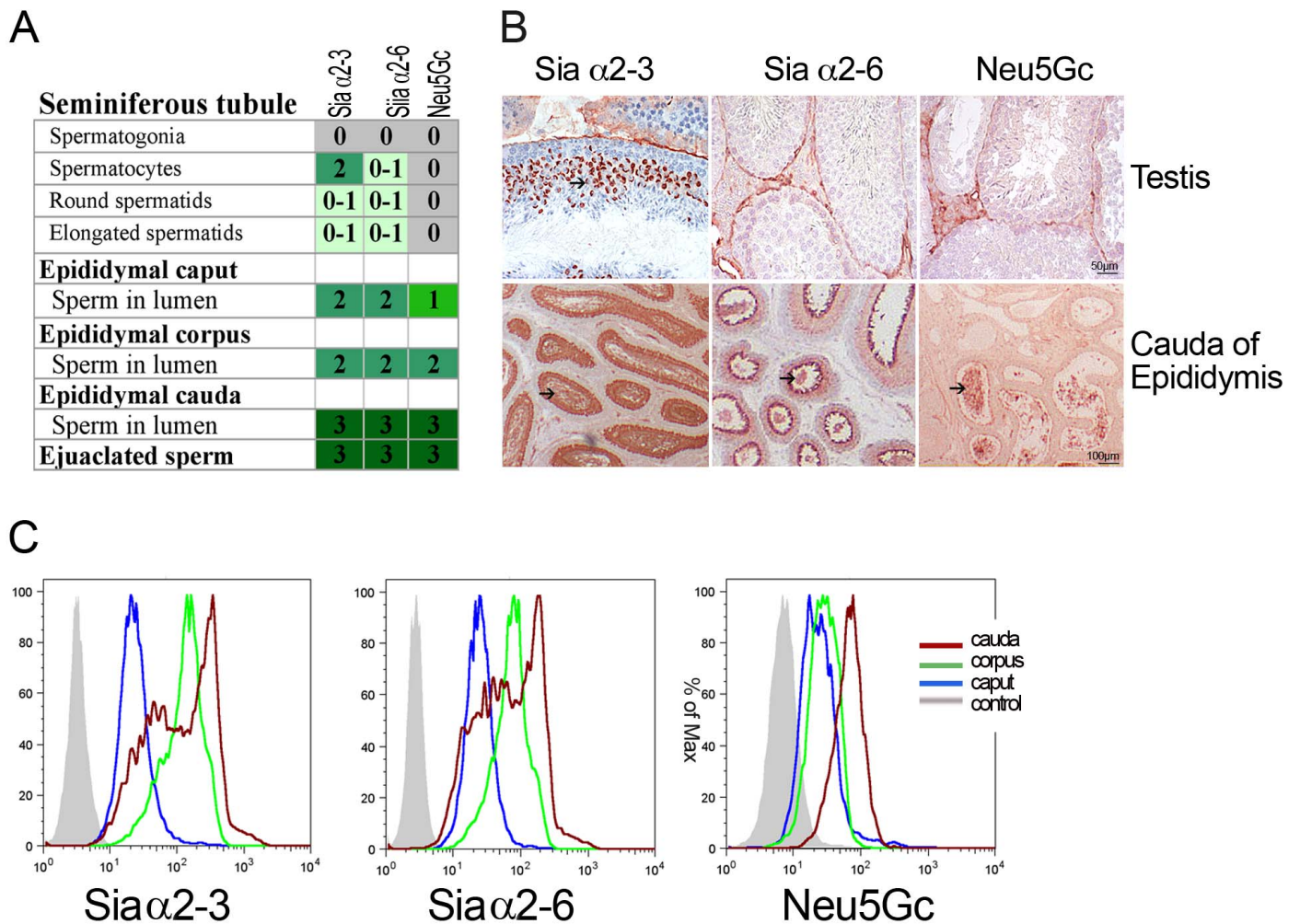


FIG. 1. Sialome profile of sperm from testis to ejaculated sperm. **A**) Summary of sperm sialylation status during different developmental stages by lectin histology and anti-Neu5Gc immunohistochemical staining. Except for the presence of Sia  $\alpha$ 2-3 on spermatocytes, incremental sialylation of other types from spermatogonia to ejaculated sperm. **B**) Sia  $\alpha$ 2-3 presence on spermatocytes and Sia  $\alpha$ 2-3/6 and Neu5Gc presence on sperm in cauda of epididymis. Frozen sections of testis and epididymis of WT male mice were fixed with acetone; incubated with SNA, MAL-II, and anti-Neu5Gc antibody as primary antibody and HRP-anti-streptavidin and HRP-anti-chicken antibody as secondary antibody; and finally developed with AEC substrate chromogen. Typical samples were chosen to show the presence of Sia  $\alpha$ 2-3 presence on spermatocytes and Sia  $\alpha$ 2-3/6 and Neu5Gc presence on sperm in cauda of epididymis (black arrows). Bars = 50  $\mu$ m (top) and 500  $\mu$ m (bottom). **C**) Quantification of incremental sialome of Sia  $\alpha$ 2-3/6 and Neu5Gc on sperm from caput to cauda of the epididymis. Sperm samples collected from caput to cauda of epididymis were fixed by PFA and detected for Sia  $\alpha$ 2-3/6 lectin staining and anti-Neu5Gc antibodies staining via flow cytometry. Sialylation levels increased from the caput to cauda of epididymis in male mice.

mice, which contains CD52 decorated with Neu5Gc. We measured the change in sialylation by staining the sperm with anti-Neu5Gc and anti-CD52 antibodies on Western blot and by flow cytometry. Neu5Gc detected on the *Cmah*<sup>-/-</sup> sperm that were coincubated with WT mice SVF would reflect Sia from SVF.

We found striking differences in staining of Western blots for a glycoprotein of  $\sim$ 20 kDa between epididymal sperm, sperm after coincubation with SVF, and SVF alone (Fig. 4A). We found that CD52 from SVF was added to epididymal sperm, and that Sia  $\alpha$ 2-3/6 and Neu5Gc were acquired at the same time (Fig. 4A).

The double staining with anti-CD52 and anti-Neu5Gc antibodies confirmed the addition of sialylated CD52 (Fig. 4B). Lectin staining indicates that more  $\alpha$ 2-6 linked Sia was obtained with CD52 (Fig. 4C). Neu5Gc was clearly acquired by *Cmah*<sup>-/-</sup> sperm after incubation with WT SVF, but these sperm exhibited an overall reduced sialome compared to WT sperm (Fig. 4, D and E). The Neu5Gc signal is predominantly localized to the sperm head; this finding suggests that

sialylation acquired by GPI-anchored CD52 and ganglioside during ejaculation mostly affects that region of the sperm. It appears that final Sia addition to the ejaculated sperm disproportionately involves the sperm head.

#### *Sperm Sialylation Protects Sperm from Female Macrophage Attack*

The mechanism by which a mature sialome protects sperm from female immune response in the uterus remains unknown. The millionfold reduction in sperm numbers in the uterus is mostly due to the action of female leukocytes. Although the mechanism of phagocytosis of sperm by uterine leukocytes remains unclear, here we chose to study direct recognition and phagocytosis by macrophages. We retrieved leukocytes from female mice 15 h after mating, when large numbers of leukocytes have infiltrated the uterus [31], and used sperm of *Gfp*<sup>+/+</sup> male mice (Fig. 5A), which have green fluorescent midpieces, as a tracer for monitoring phagocytosis by macrophages. Additionally, we used AUS treatment to cleave

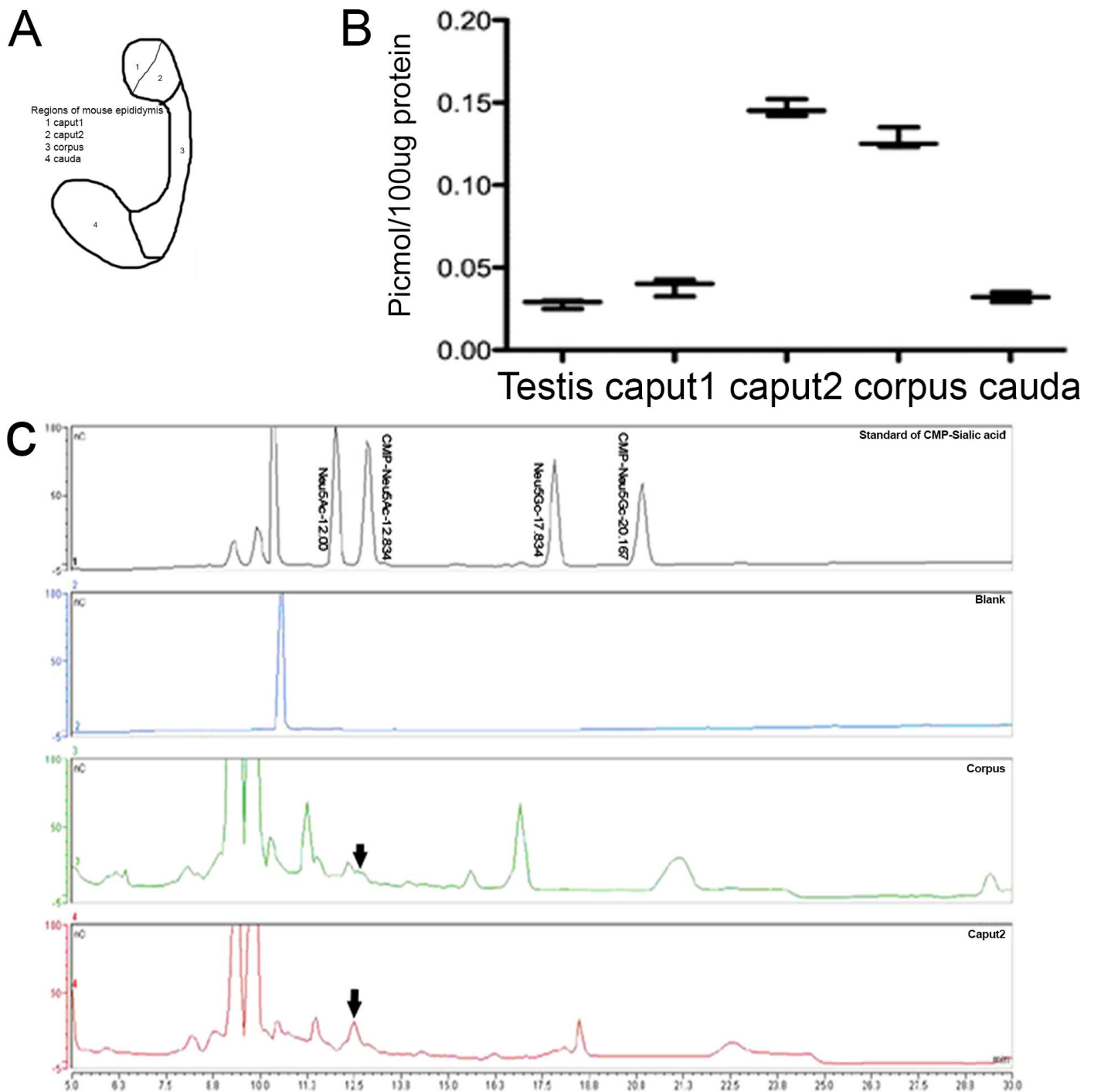


FIG. 2. Determination of CMP-Sia in epididymal fluid. **A)** Simple scheme of the four regions of mouse epididymis based on literatures and the anatomy of male mice. Obvious blood vessels were observed between caput 1 and caput 2 in operation. **B)** Quantification of CMP-Neu5Ac level in the regional epididymal fluids. Regional epididymal fluids were detected by HPAEC-PAD. The results were presented as picomole CMP-Neu5Ac per 100  $\mu$ g total protein. The CMP-Neu5Ac levels in caput 2 and corpus were higher than in testis, caput 1, and cauda. The data are expressed as mean  $\pm$  SEM (n = 5). **C)** CMP-Neu5Ac peaks in the representative regional epididymal fluids by HPAEC-PAD (CMP-Sia standard as positive control and distilled water as negative control). The peak of CMP-Neu5Ac is pointed out by black arrows.

most of the Sias from the sperm membrane and tested the effect of such a loss of Sias. Paradoxically, there is very low phagocytosis of the epididymal sperm when compared with that of sperm coincubated with SVF, which had a 46% phagocytosis rate (Fig. 5, B and C). If Sias were removed by AUS treatment from sperm coincubated with SVF, phagocytosis increased even further (57%; Fig. 5, B and C). Factors from SVF thus both increase the likelihood of sperm being

phagocytosed by uterine macrophages and mitigate that risk by contributing sialoglycoproteins.

## DISCUSSION

As the most common terminal monosaccharides on mammalian cells, Sias form the “molecular frontier of the glycocalyx.” This predisposes this family of sugars for playing

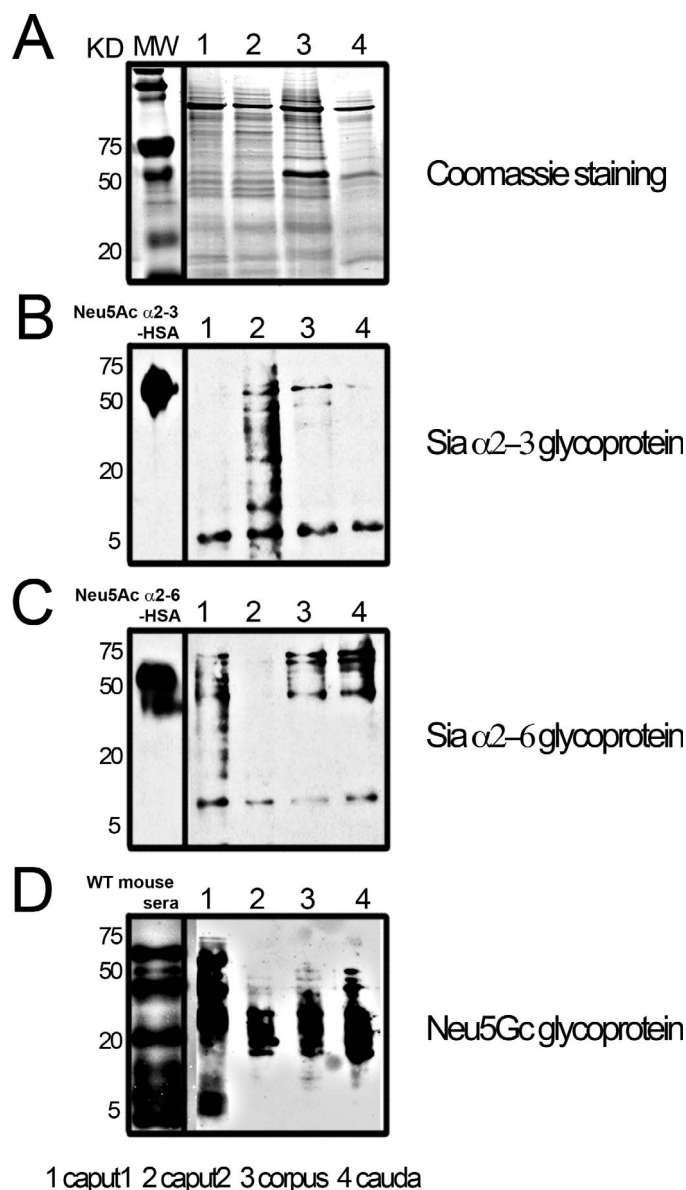


FIG. 3. Detection of sialoglycoprotein in epididymal fluid from different epididymal segments. **A**) Contents and semiquantification of the regional epididymal fluid on reduced SDS-PAGE gel by Coomassie staining, loading, 10  $\mu$ g protein/lane. Sialoglycoproteins were found throughout the epididymis; the amount and composition varied between fluids from different epididymal segments. **B**) Sia  $\alpha$ 2-3 glycoproteins in regional epididymal fluids. Sia  $\alpha$ 2-3 glycoproteins in the samples were detected by MAL-II-specific binding (loading: HSA, 100 ng; others, 10  $\mu$ g protein/lane). HSA was used as positive control in the first lane. **C**) Sia  $\alpha$ 2-6 glycoproteins in the regional epididymal fluids. Sia  $\alpha$ 2-6 glycoproteins in the samples were detected by SNA-specific binding (loading: HSA, 100 ng; others, 10  $\mu$ g protein/lane). HSA was used as positive control in the first lane. **D**) Neu5Gc-glycoproteins in regional epididymal fluids. Neu5Gc-glycoproteins in the samples were detected by anti-Neu5Gc antibody (loading: WT mouse sera, 100  $\mu$ g; others, 10  $\mu$ g protein/lane). Mouse serum was used as positive control in the first lane.

multiple roles in cellular recognition processes. As foreign cells, mammalian sperm face numerous challenges during their transit through the female reproductive tract en route to fertilization [13]. That insemination with hundreds of millions of individual sperm is required to fertilize one or a few ova is likely a good indication of the magnitude of adversity faced by sperm. Surviving the onslaught of female immune factors is an

absolute prerequisite for sperm to reach the upper oviduct for fertilization. The down-regulation of major histocompatibility complex (MHC) class I molecules on mature sperm allows them to avoid histocompatibility-based immune responses by female MHC I-restricted cytotoxic T cells. Abundant sialylation likely facilitates tolerance by female innate pattern-recognition molecules, both secreted molecules such as complements (e.g., factor H, which binds to Sia and prevents complement deposition) [32] and cell-bound molecules on female leukocytes (e.g., Sia-recognizing immunoglobulin superfamily lectins [Siglecs], which bind to Sia and can inhibit immune cell activation) [33]. In addition, decoration with terminal Sia may play a role in masking potential antigenic sperm molecules until after sperm have undergone capacitation and sperm have approached the egg and its vestment. Such masking has been suggested for estrogen and *N*-acetyl-5-methoxytryptamine receptors on the sperm midpiece, which contribute to the regulation of sperm motility by inhibiting the breakdown of ATP [34, 35]. Similarly, unmasked tyrosine phosphorylated chaperone proteins overlaying the acrosome of capacitated sperm might mediate sperm zona binding [36]. Masking of potent sperm antigens such as ph20 by highly sialylated DEFB126 has also been demonstrated [28].

Changes in sperm sialylation level en route to fertilization have been reported for several decades (Table 1); however, the mechanisms involved remain unknown. At the beginning of spermatogenesis, spermatogonia, or immature germ cells, divide mitotically to produce diploid cells termed spermatocytes. Subsequently, after meiotic cell division, spermatids are released from the testes into the epididymis, where they undergo a process of maturation and ultimately acquire motility. Sperm surfaces are further modified by absorption of coating molecules from seminal fluid during ejaculation.

Regarding the gain of Sia during sperm development, a recent study reported Sia decoration on sperm during spermatogenesis in the testes [37], and earlier studies have reported sperm acquire Sia during epididymal transit and maturation, a process that involves sialylation by luminal sialyltransferase particularly in the caput epididymis [8, 9, 38]. Additional increases in sperm sialome were shown to occur via incorporation of secreted GPI-anchored sialoglycoproteins, especially CD52, and also incorporation of gangliosides (Sia containing glycosphingolipids) from seminal fluid during ejaculation. We found that there was free CMP-Neu5Ac (the donor sugar nucleotide used by sialyltransferases to add Sias to a glycan chain) as well as secreted sialoglycopeptides with  $\alpha$ 2-3- and  $\alpha$ 2-6-linked Sias in different regions of the lumen of the epididymis. This indicates that sperm sialome gain involves sialyltransferase activity and incorporation of GPI-anchored sialoglycopeptide (Figs. 2 and 3). Meanwhile, sperm acquire the bulk of the GPI-anchored sialoglycopeptide CD52 from seminal fluid. Incubating sperm with SVF resulted in increases of  $\alpha$ 2-3- and  $\alpha$ 2-6-linked Sias, and Neu5Gc on glycopeptides or ganglioside (Fig. 4). In conclusion, the mechanism of sialylation on sperm in male genital tract is shown in Figure 6.

It was reported that in female golden hamsters, the highest number of leukocytes (average 1 497 000) was found 12–14 h after mating with a fertile male, mainly because of the presence of semen itself and the large numbers of spermatozoa in the uterus. The rapid disappearance of spermatozoa from the uterus on the first day following mating is ascribed to the phagocytotic action of leucocytes and drainage through the cervix, and the mechanism of how leucocytes recognize the sperm remains unclear. On the other hand, it is important to maintain an adequate number of incapacitated sperm that can move into the oviduct to meet the ovum. Therefore, protecting

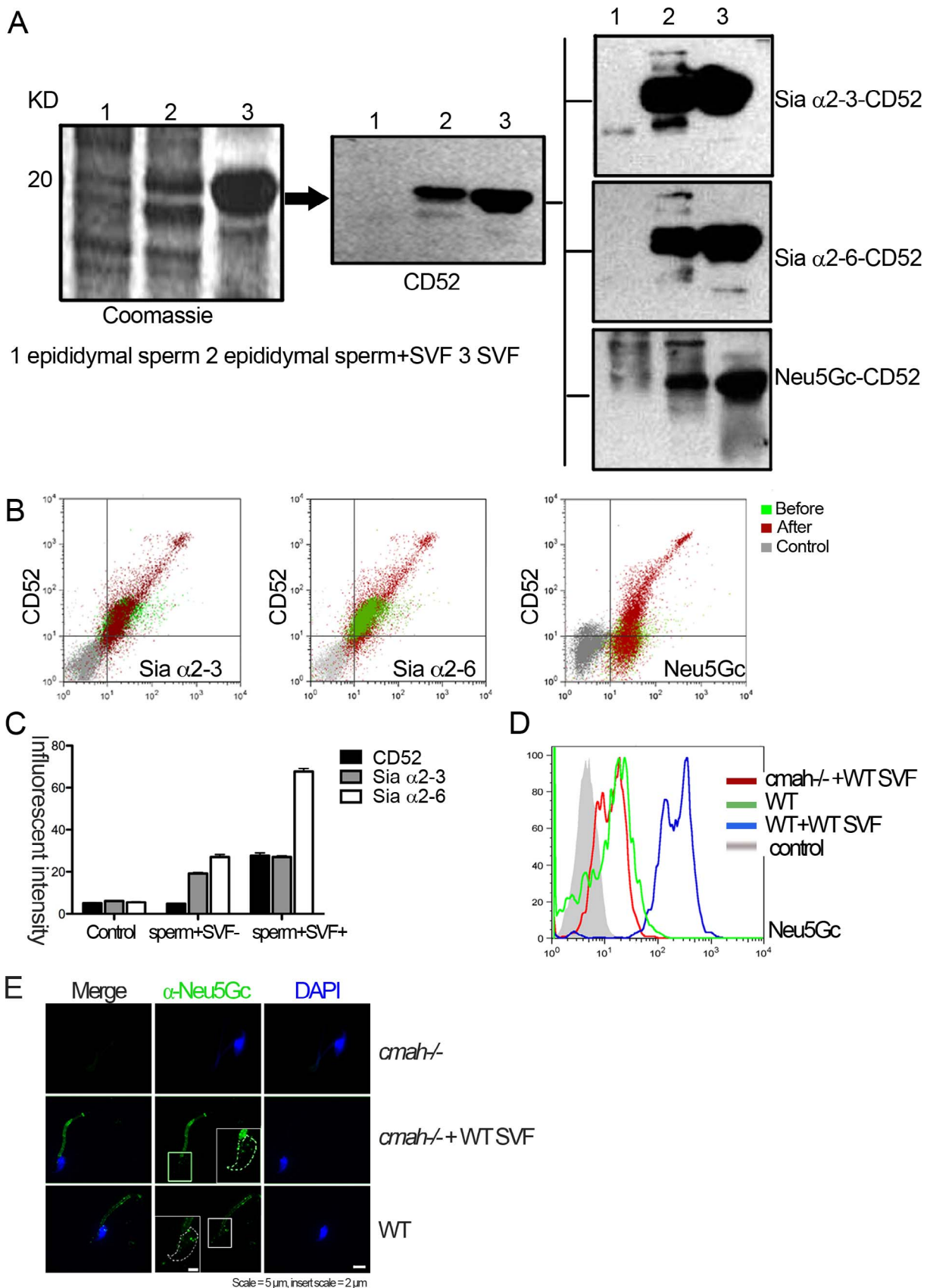


FIG. 4. Sialylation of epididymal sperm by exposure to SVF. **A**) Samples: 1, epididymal sperm; 2, epididymal sperm coincubated with SVF; 3, SVF. Left panel: sperm sialoglycoproteins in the samples. Proteins around 20 kDa molecular mass were presented on SDS-PAGE gel by Coomassie staining (loading: 10  $\mu$ g protein/lane). Middle panel: presence of CD52 in the samples. Samples were blotted with anti-CD52 antibody (loading: 10  $\mu$ g/lane). Right panel: detection of Sia  $\alpha$ 2-3/6 and Neu5Gc linked with CD52 in the samples. Proteins were detected with specific lectin binding and anti-Neu5Gc antibody (loading: for Sia  $\alpha$ 2-3/6, 10  $\mu$ g/lane; for Neu5Gc, 2  $\mu$ g/lane). **B**) Expression of Sia  $\alpha$ 2-3/6 and Neu5Gc on CD52 in sperm samples before (green dots) and



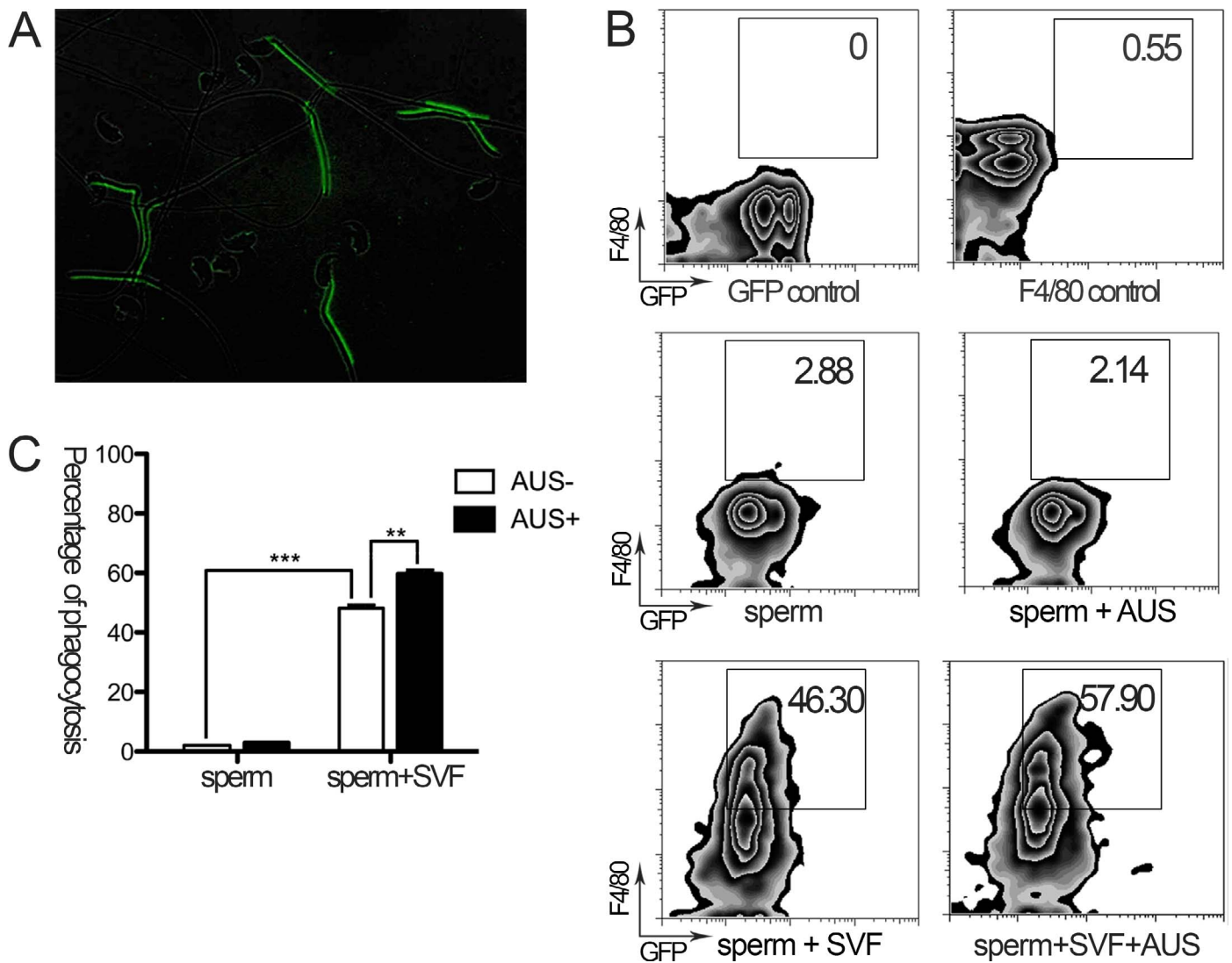


FIG. 5. Evidence for the role of sialylation and its protection from phagocytosis by macrophages. **A)** Distribution of green fluorescence on *Gfp*<sup>+/+</sup> mouse sperm. The sperm of transgenic *Gfp*<sup>+/+</sup> mouse were directly observed under fluorescent microscope. The green fluorescence was mainly concentrated in the middle section of the sperm. **B)** Detection of phagocytosed *Gfp*<sup>+/+</sup> sperm by fluorescence-activated cell sorting (FACS). (*Gfp* positive control: *Gfp*<sup>+/+</sup> sperm; F4/80 positive control: macrophage). AUS treatment of sperm: 15 U/100 000 sperm cells; 10 000 cells were counted for each group. The quantized phagocytosis data are presented in each frame. Low phagocytosis of the epididymal sperm was observed comparing with that of the sperm coincubated with SVF. **C)** Quantification of phagocytosis of sperm by macrophage according to the FACS data. The data are expressed as mean  $\pm$  SEM ( $n = 5$ ;  $**P < 0.01$ ;  $***P < 0.001$ ).

TABLE 1. Evidence for modulation of sperm Sia content.

Mechanism	Method	Evidence	Location of gain	Species	Author [reference]
Transfer from CMP-Sia	Coincubation sialyltransferase with CMP-Sia and sperm	Indirect	Epididymis	Rat	Bernal et al. [12]
Transfer of gangliosides	Thin layer chromatography	Indirect	Ejaculation	Human	Ritter et al. [22]
Sialoglycoprotein	Western blot	Indirect	Epididymis	Mouse	This study
Transfer from CMP-Sia	HPAEC-PAD	Direct	Epididymis	Mouse	This study
Transfer of GPI-sialoglycoprotein	Western blot/FACS/coincubation with SVF	Direct	Vas deferens	Mouse	This study

after (red dots) coincubation with WT SVF. The samples were analyzed by fluorescence-activated cell sorting (FACS) using double staining (Negative control signals: gray dots). Fifty thousand sperm cells were counted for each group. **C)** Quantification of the different sialylation linkage (Sia  $\alpha 2-3/6$ ) on CD52 with fluorescent intensity based on the FACS data. The data are expressed as mean  $\pm$  SEM ( $n = 5$ ). **D)** Evidence of analyzed sialylation on *Cmah*<sup>-/-</sup> mouse epididymal sperm coincubated with WT SVF detected by anti-Neu5Gc antibody by FACS. Fifty thousand sperm cells were counted for each group. **E)** Image of analyzed sialylation on *Cmah*<sup>-/-</sup> mouse epididymal sperm coincubated with WT SVF detected by anti-Neu5Gc antibody (green), DAPI (blue) counterstained. Bars = 5  $\mu$ m and 2  $\mu$ m (inset).

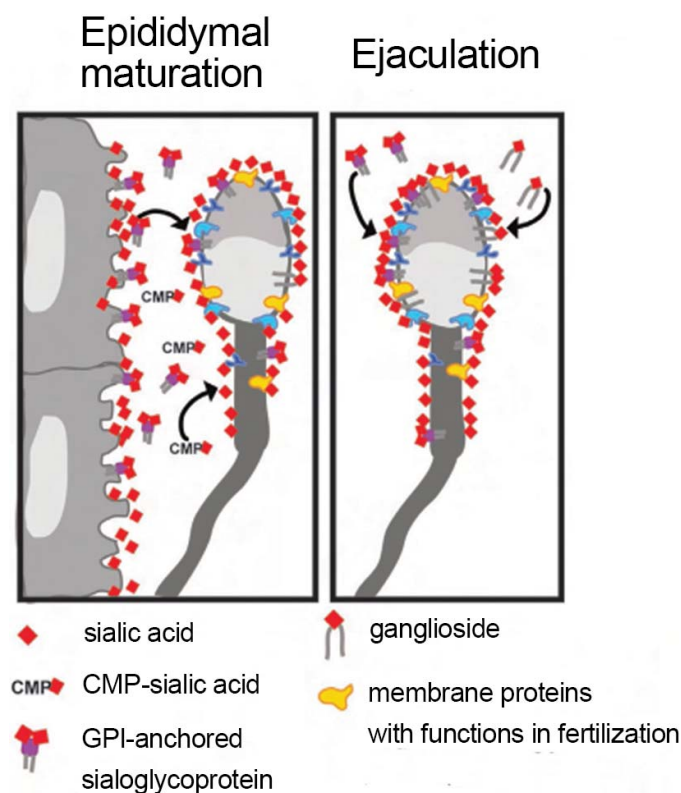


FIG. 6. Sperm gain Sias via incorporation of CMP-sialic acid, GPI-anchored sialoglycoprotein, and ganglioside in male genital tract. The diagram was drawn based on our data and other available research data.

sperm from excessive phagocytosis by leucocytes is crucial. In our study (Fig. 5), it was identified that epididymal sperm without complete sialylation were not recognized by uterine macrophages. It was reported that the 54-kDa sialoglycoprotein with the aid of Sia residues was implicated in the protection of sperm from phagocytosis [20]. However, epididymal sperm that were desialylated by treating with AUS, which partly cleaves the Sia from sialoglycoprotein and ganglioside, underwent phagocytosis at a much higher rate than sperm incubated with SVF without AUS treatment. This indicates that intact sialylation plays a key role for the recognition of sperm by macrophages or neutrophils. That the desialylation of sperm increases phagocytosis by macrophages suggests sialylation might mask some molecules to protect the sperm from being chased by leucocytes.

In our study, we profiled sialylation addition during sperm maturation and provide evidence that the sialome may contribute to the protection of sperm from female uterine immune responses. On the other side, our reported study found that sialidases are present on the surface of mouse and human sperm, which are activated during capacitation [39]. The coexpression of sialyltransferase and sialidase on sperm might give some clue to explore how and why Sias change in the lifespan of sperm, similarly to the “yin and yang” mechanism, to keep the balance of sialylation and desialylation. Future studies should focus on further characterizing the mechanism of sialylation and its contribution to sperm function and immune evasion.

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