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Authors

Teclé, Eillen
Reynoso, Hector Sequoyah
Wang, Ruixuan
et al.

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The female reproductive tract contains multiple innate sialic acid-binding immunoglobulin-like lectins (Siglecs) that facilitate sperm survival

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Eillen Teclé¹, Hector Sequoyah Reynoso¹, Ruixuan Wang, and  Pascal Gagneux²

From the Department of Pathology, Glycobiology Research and Training Center, University of California San Diego, San Diego, California 92093

Edited by Luke O'Neill

A sperm that fertilizes an egg has successfully survived multiple checkpoints within the female reproductive tract, termed pre-fertilization events. The leukocytic response is a pre-fertilization event in which sperm trigger an immune response that promotes homing of circulating leukocytes to the uterine lumen to destroy most sperm. Various glycoconjugates decorate the sperm surface, including sialic acids, which are abundant at the sperm surface where they cap most glycan chains and regulate sperm migration through cervical mucus, formation of the sperm oviductal reservoir, and sperm capacitation. However, the role of sperm-associated sialic acids in the leukocytic reaction remains unknown. The cognate endogenous binding partners of sialic acids, sialic acid-binding immunoglobulin-like lectins (Siglecs) play a pivotal role in regulating many immune responses. Here we investigated whether sperm-associated sialic acids inhibit activation of neutrophils, one of the major immune cells involved in the leukocytic reaction. We used *in vitro* interactions between sperm and neutrophils as well as binding assays between sperm and recombinant Siglec-Fc chimeric proteins to measure interactions. Moreover, we examined whether Siglecs are expressed on human and mouse endometria, which have a role in initiating the leukocytic reaction. Surprisingly less sialylated, capacitated, sperm did not increase neutrophil activation *in vitro*. However, we observed expression of several Siglecs on the endometrium and that these receptors interact with sialylated sperm. Our results indicate that sperm sialic acids may interact with endometrial Siglecs and that these interactions facilitate sperm survival in the face of female immunity.

Like all living cells, sperm are covered in a glycocalyx, a complex “sugar coat” consisting of diverse glycan chains conjugated

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This article contains Figs. S1–S6.

¹ Both authors contributed equally to this work.

² To whom correspondence should be addressed. E-mail: pgagneux@ucsd.edu.

to cell-surface proteins and lipids (Fig. 1a). Unlike most cells, mammalian sperm are introduced into the reproductive tract of a different individual where they have to survive and remain functional until they fertilize an egg in the ampulla of the fallopian tubes (1). This feat is made more difficult because of the presence of large numbers of immune cells and secretions in the female reproductive tract (2–6). The female tract is well-equipped with innate immune receptors including several Toll-like receptors (7) that target potentially harmful, nonself molecules such as pathogen-associated molecular patterns. In contrast, previous work has shown that components of the glycocalyx can function as self-associated molecular patterns (SAMPs)³ by interacting with inhibitory receptors on immune cells (8). Thus, SAMPs defined by constituents of the sperm glycocalyx may facilitate sperm survival in the face of female immunity by binding to glycan-recognizing receptors along the female reproductive tract and thus contribute to successful fertilization (9). A common and well-documented determinant of SAMPs present within the sperm glycocalyx is the terminal monosaccharide sialic acid. Sialic acid is a nine-carbon backbone, amino sugar located at the terminal end of gangliosides, N-glycans and O-glycans and is present in high abundance, tens to hundreds of millions of molecules per cell (10).

Sialic acids can modulate immune response by binding to sialic acid-binding immunoglobulin-like lectins (Siglecs), which are primarily expressed by leukocytes (11, 12) and also by binding to factor H of complement (13)(Fig. 1b). All Siglecs contain one or more Ig domains (C2-set) and a N-terminal sialic acid-binding domain (V-set). Inhibitory Siglecs contain cytosolic immunoreceptor tyrosine-based inhibition motif (ITIM) and ITIM-like domains (ITIM-L) (14). The ITIM and ITAM-like domains of inhibitory Siglecs are phosphorylated by a Src family tyrosine kinase allowing for recruitment of SHP-1 (Src homology region 2 domain-containing phosphatase-1) resulting in signaling that inhibits activation of an immune

³ The abbreviations used are: SAMP, self-associated molecular pattern; DAMP, danger-associated molecular pattern; C, capacitated; IHC, immunohistochemistry; ITIM, immunoreceptor tyrosine-based inhibition motif; ITAM, immunoreceptor tyrosine-based activation motif; JC-1, 5',6',6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide; NC, uncapacitated; Siglec, sialic acid-binding immunoglobulin-like lectin; TBP, TATA-binding protein; 4MU-NANA, 4-methylumbelliferyl α DN-acetylneuraminic acid; ROS, reactive oxygen species; NET, neutrophil extracellular trap; PMA, phorbol 12-myristate 13-acetate; qPCR, quantitative PCR; SHP-1, Src homology region 2 domain-containing phosphatase-1.

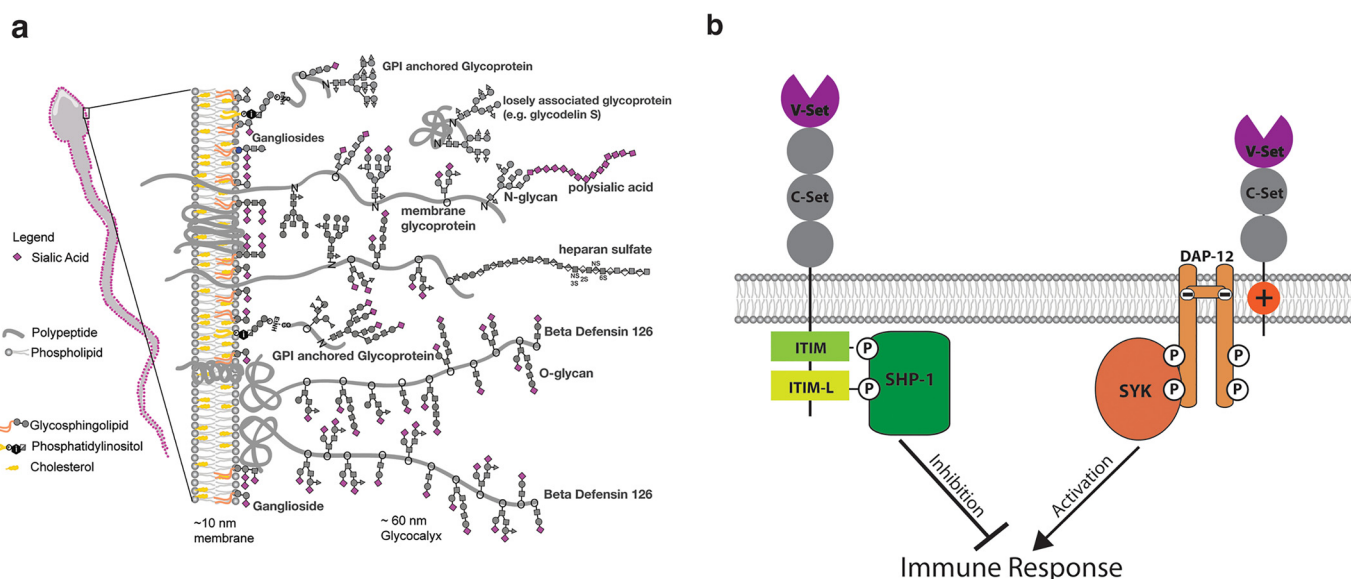


Figure 1. The sperm glycocalyx and siglec signaling. *a*, a schematic of the glycocalyx of mammalian ejaculated sperm. Sialic acid is presented as purple diamonds. Modified from Ref. 1. *b*, Siglecs and downstream signaling. A schematic of an inhibitory Siglec (left) and activating Siglec (right) are shown. After binding a sialic acid ligand, via the V-set domain, Siglecs can modulate an immune response by downstream signaling pathways. Phosphorylation the ITIM and ITIM-L domains of inhibitory Siglecs by SHP-1 initiates a signaling cascade resulting in immune inhibition. Activating Siglecs bind to DAP12 via their transmembrane ITAM domains and initiate a signaling cascade resulting in immune activation.

response (15). In contrast, activating Siglecs contain a transmembrane immunoreceptor tyrosine-based activation motif (ITAM) domain. Via the ITAM, activating Siglecs bind to DAP12, which is phosphorylated by a Src family tyrosine kinase allowing for recruitment of SYK resulting in a signaling cascade that promotes activation of an immune response (15).

The female reproductive tract has been previously shown to have immune surveillance that is hormonally regulated to promote fertility while still providing antimicrobial resistance (16). Sperm in the uterus trigger the leukocytic reaction, a rapid-onset immunological response that promotes homing of circulating leukocytes to the uterine lumen (3, 4). This results in the active destruction of the majority of sperm. It is not fully understood how some sperm survive the leukocytic reaction. It is unknown whether Siglecs play a role in the modulation of the immune system during insemination and if Siglecs are present within the female reproductive tract.

However, the presence of sialic acids appears to be important for sperm function. For instance, sialic acids on highly sialylated betadefensin 126 is absolutely required for human sperm to penetrate cervical mucus and for sperm to adhere to the oviductal epithelium before capacitation occurs (17). Capacitation refers to several cellular processes that occur in mammalian sperm before they are competent to fertilize an egg, including an increase in mitochondrial activity to sustain vigorous motility (18–20). Interestingly, previous reports indicate that mammalian sperm partially shed sialic acids during capacitation (19). Although sialic acids are known to be critical for sperm migration, it is unclear whether they also facilitate sperm survival within the female reproductive tract.

In vivo, biologically relevant capacitation is thought to occur in the isthmus of the oviduct. In the isthmus, sperm adhere to the oviductal epithelium in a sialic acid-dependent manner forming the mammalian sperm reservoir (23). Sperm detach from the oviductal epithelium when capacitation is induced

and swim toward the egg in the ampulla of the oviduct (23). One successful sperm will acrosome react, digest the protective vestments of the egg, bind to the zona pellucida and upon reaching the perivitelline space, fuse with the egg membrane, resulting in fertilization (21). The oviduct contains a very different immune cell repertoire and it is assumed that sperm, which make it there, are under much reduced immune threat (24). A minority of sperm successfully pass the utero-tubal junction leading to the ovulatory oviduct (21) after they have traveled through the uterus and survived the leukocytic reaction. The leukocytic reaction is a pre-fertilization event in which sperm arriving in the uterus trigger an immunological response that promotes homing of circulating leukocytes to the uterine lumen resulting in the active destruction of the majority of sperm (3, 4). Of the few sperm that survive the onslaught of leukocytes in the uterus, even fewer successfully migrate to the isthmus of the oviduct (22).

We hypothesize that sperm sialic acids and female-expressed Siglecs may modulate the leukocytic reaction and promote sperm survival within the female reproductive tract. Indeed, a recent report has shown that sialic acids on sperm are protective against phagocytosis by macrophages, *ex vivo* (25). To begin to address this, we investigated sialic acid-dependent interactions between sperm and isolated neutrophils *in vitro*. Neutrophils were chosen because they represent the most abundant immune cell in the human leukocytic reaction following insemination (4) and have documented expression of inhibitory Siglecs-5 and Siglec-9 (26, 27). As capacitation is associated with the loss of sialic acid, which are required to engage inhibitory Siglecs, we investigated via several measures of neutrophil reactivity whether capacitated (C) sperm invoke greater reactivity than noncapacitated (NC) sperm.

Within the female reproductive tract, the endometrium has an established role in responding to and promoting the destruction of pathogens and/or sperm in the uterus (28). However, at least some sperm must survive within the female reproductive

Endometrial Siglecs

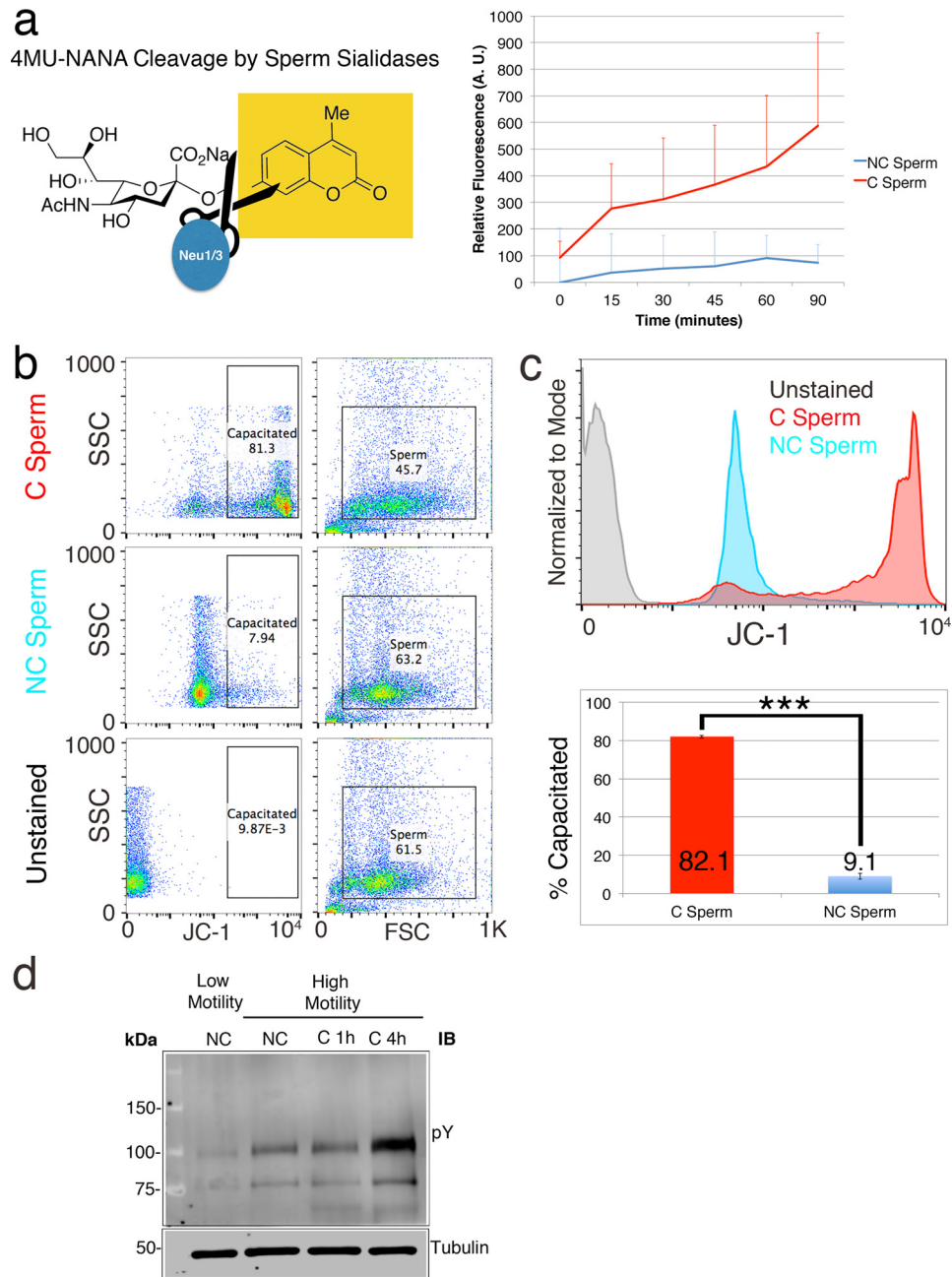


Figure 2. Confirmation of sperm capacitation and sialic acid shedding. Sperm co-incubated with the fluorogenic sialic acid analog 4-MU-NANA were exposed to a noncapacitating or capacitating buffer. Only sperm in the capacitating buffer show an immediate increase in fluorescence (a), indicative of rapidly activated sperm sialidases. Sperm capacitation was confirmed by using the fluorescent molecule JC-1 to probe for increased mitochondrial activity. The majority of sperm incubated under capacitating conditions show an increase in JC-1 staining (b and c), compared with sperm in noncapacitating conditions (~81 versus ~10%, respectively). Staining with anti-phosphotyrosine antibody on Western blotting shows an increase of phosphorylation between low motility (LM) and high motility (HM) human sperm and with increased duration (1 versus 4 h) of *in vitro* exposure to capacitating conditions (d).

tract during the fertile window to achieve fertilization. Therefore, we examined the expression of Siglecs in the human and mouse endometrium. Finally, we tested sialic acid-dependent binding of sperm by Siglecs using recombinant, chimeric Siglec-Fc proteins of Siglecs identified in the endometrium.

Results

Sialidases are immediately activated on sperm exposed to capacitating conditions

We measured the temporal dynamics of sialic acid loss in capacitating sperm using a reporter molecule 4-MU-NANA.

Cleavage of the sialic acid analog NANA results in the emission of fluorescence from the separated 4-MU molecule. Sperm were incubated together with 4MU-NANA in either noncapacitating or capacitating buffers, and the resulting changes in fluorescence were measured via plate reader. Sperm show an increase in fluorescence immediately upon exposure to capacitating conditions, indicative of sialidase activity (Fig. 2a). Non-capacitated sperm do not show any increase in fluorescence. The percentage of capacitated sperm following *in vitro* capacitation was measured via flow cytometry by probing for an increase in mitochondrial activity using the mitochondrial

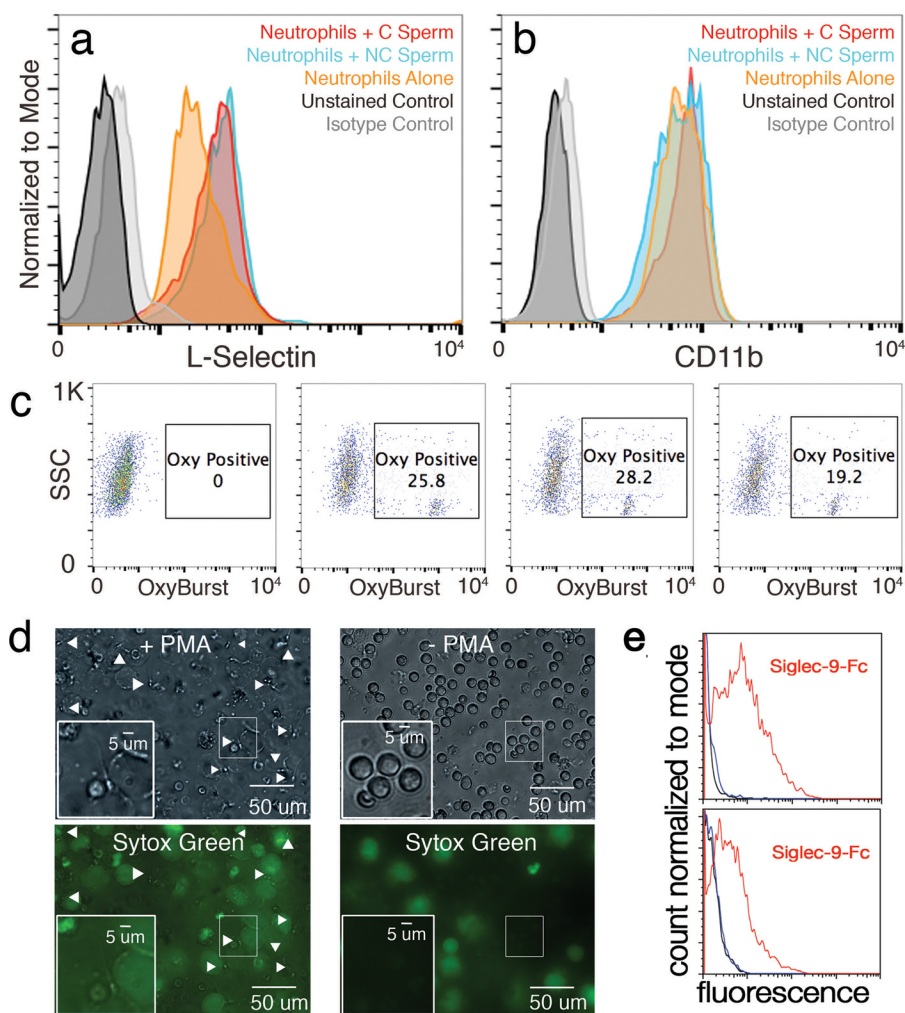


Figure 3. Impact of sperm on neutrophil activation. Flow cytometry analysis of the expression of several neutrophil reactivity markers (a–c). Neutrophils co-incubated with NC or C sperm were stained with antibodies targeting several markers of neutrophil activation and measured using flow cytometry. Co-incubation of isolated human leukocytes with NC or C sperm show no additional shedding of the neutrophil quiescence marker L-selection when compared with leukocytes alone (a). Isolated human neutrophils co-incubated with C or NC sperm show similar CD11b expression compared with isolated leukocytes alone (b). Isolated human neutrophils co-incubated with C sperm show a nonsignificant decrease in the percentage of OxyBURST-positive cells (positivity indicates an increase in intracellular reactive oxygen species) compared with isolated neutrophils alone or neutrophils co-incubated with NC sperm (c). PMA-induced isolated human neutrophils trigger NET formation and capture live NC and C sperm (d). Captured sperm are indicated by white arrowheads. e, sperm from two different donors stained with Siglec-Fc chimeric recombinant protein: black is control, blue is Siglec-5::Fc, red is Siglec-9::Fc.

membrane potential probe JC-1 (29). The majority of C sperm showed a robust increase in JC-1 compared with NC sperm (Fig. 2, b and c) and staining of Western blots with antiphosphotyrosine antibody confirms capacitation (Fig. 2d).

Capacitated sperm do not increase neutrophil activation *in vitro*

We are using *in vitro* capacitation as a biologically relevant way of shedding sialic acids from sperm to test the effect of reduced sialylation on interactions with leukocytes. We investigated the differential impact of sperm with more (NC sperm) or less sialic acid (C sperm) exposure on neutrophil activation *in vitro* using four different measures: L-selectin shedding, expression of CD11b, reactive oxygen species (ROS) production, and NET formation. First, activation of human neutrophils was determined by shedding of the quiescence marker L-selectin after exposure to C and NC human sperm. The amount of L-selectin present on neutrophils after incubation *in vitro*

was probed using L-selectin antibodies and analyzed via flow cytometry. In one trial, exposure to both C and NC sperm preserved L-selectin as compared with untreated neutrophil controls (Fig. 3a), but this effect was not consistent (Fig. S1). We next determined the reactivity state of neutrophils exposed to C and NC sperm by assessing levels of the activation marker CD11b. Anti-CD11b reactivity was measured via flow cytometry. C sperm did not generate activation of neutrophils *in vitro*, except for a small subpopulation in 1 of 3 experiments (Fig. S2). In 1 of 3 replicates, C sperm generated the weakest activation response in neutrophils as compared with that of NC sperm and untreated neutrophils (Fig. 3b). Neutrophils can perform their microbicidal function via the creation of ROS following endocytosis of foreign microbes (30). Thus, we investigated the generation of reactive oxygen species in neutrophils after exposure to C and NC sperm as an alternate measure of neutrophil activation. ROS production was probed using OxyBURST and measured via flow cytometry. C sperm did not generate a strong

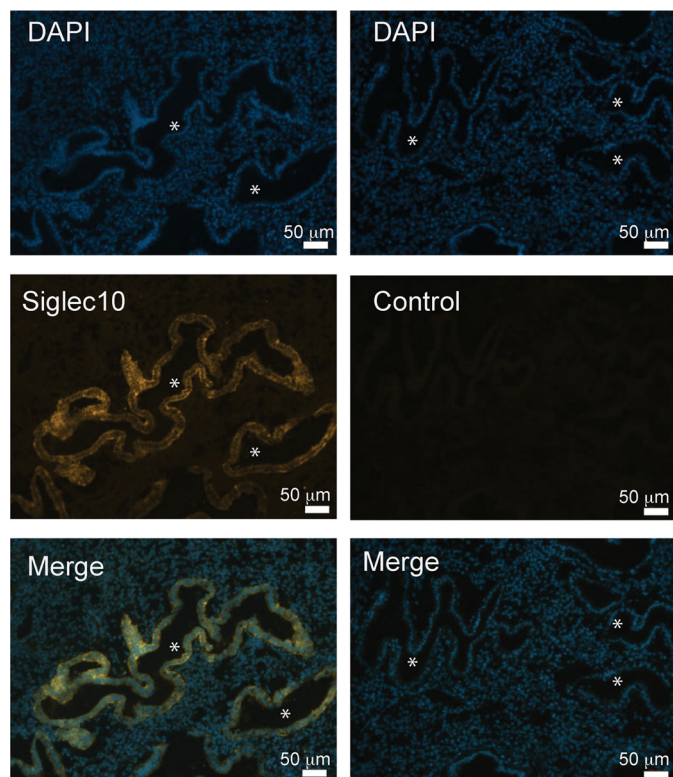


Figure 4. Siglec-10 is expressed in the human endometrium. Immunofluorescence staining of Siglec-10 (orange) and all nuclei (4',6-diamidino-2-phenylindole, DAPI). Siglec-10 expression is observed exclusively in the endometrium and not in the underlying stroma. Asterisks indicate the uterine lumen. Images are representative for a sample of $n = 7$.

increase in ROS production by neutrophils (19.2% OxyBURST positive) compared with that of NC sperm (28.2%) and untreated neutrophils (25.8%, Fig. 3c). Neutrophils can also immobilize and destroy pathogenic threats via the creation of neutrophil extracellular traps (NETs) (31). Neutrophils triggered by phorbol 12-myristate 13-acetate (PMA) displayed a robust increase in NET formation that immobilized both NC and C sperm (Fig. 3d). Neutrophils that were not exposed to PMA and briefly exposed to NC or C sperm remained membrane intact and did not capture NC or C sperm. We confirmed engagement of Siglec-9 by sperm from two different donors by flow cytometry using recombinant Siglec-9-Fc chimera protein (Fig. 3e).

The human endometrium expresses Siglecs and downstream signaling proteins

Although our data clearly demonstrate that reduced levels of sperm sialic acids do not activate neutrophils *in vitro*, we decided to investigate the expression of Siglecs in the uterus.

As heavily sialylated NC sperm directly interact with the endometrium, we hypothesized that the endometrium may express Siglecs. Therefore, we probed for the expression of various Siglecs in human pre-menopausal uterine samples and found that Siglec-10 was expressed primarily by the epithelium ($n = 7$) (Fig. 4). We also screened two human endometrial cell lines, Ishikawa and HEC-1B, for cell-surface Siglec expression by flow cytometry analysis (Table 1, Fig. 5). Ishikawa and HEC-1B cell lines have been used extensively to investigate

Table 1
Siglec expression in two human endometrial cell lines

	Cell line	
	HEC-1-B	Ishikawa
Siglec-3	Absent	Absent
Siglec-5/14	Absent	Absent
Siglec-7	Absent	Absent
Siglec-9	Absent	Absent
Siglec-10	High	High
Siglec-11	Low	Low
Siglec-16	High	High

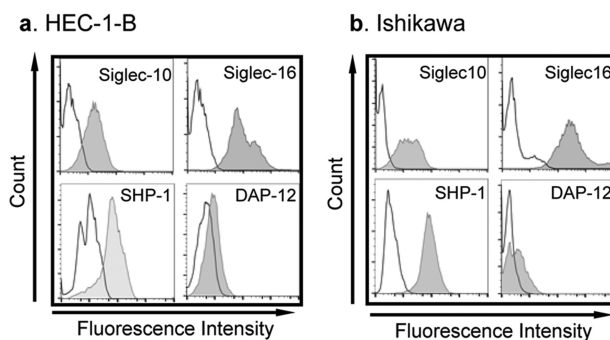


Figure 5. Siglecs and their downstream signaling proteins of Siglecs are present in endometrial cancer cell lines. Flow cytometry analysis of the expression of select Siglec and their signaling molecules in endometrial cell lines. Both HEC-1B (a) and Ishikawa (b) cell lines express Siglec-10 and Siglec-16. Both cell lines also express SHP-1 and DAP12, the downstream signaling molecules of Siglec-10 and Siglec-16, respectively.

hormonal responses of various genes or in the context of infection (32–35). These analyses revealed HEC-1B (Fig. 5a, Fig. S3) and Ishikawa (Fig. 5b, Fig. S4) express two inhibitory Siglecs, Siglec-10 and Siglec-11, as well as activating Siglecs (Siglec-16). Importantly, both SHP-1, the downstream signaling molecule of immune-inhibitory Siglec-10 and Siglec-11, and DAP12, the downstream signaling molecule of immune-activating Siglec-16, are expressed in the two endometrial cell lines (Fig. 5).

Human sperm bind Siglec-10 in a sialic acid-dependent manner

To investigate the possible role of Siglecs in the leukocytic reaction, we assessed the binding of recombinant Siglec-Fc fusion proteins expressed by the endometrium to noncapacitated human sperm. Siglec-Fc fusion proteins contain the V-set and one or more underlying C-set of a given Siglecs in-frame to the human IgG Fc region. We tested the binding of Siglec-10, -11, and -16-Fc fusion proteins to sperm. Sperm did not bind Siglec-11 or Siglec-16-Fc proteins (Fig. 6, a and b). However, sperm robustly bound Siglec-10-Fc fusion proteins (Fig. S5). In addition, this binding was sialic acid dependent as no binding was observed using a Siglec-10 mutant protein with a R120K mutation, which abrogates its sialic acid binding ability by virtue of lacking the critical arginine residue in the outermost V-set domain responsible for sialic acid binding (Fig. 6c). Furthermore, we found by flow cytometry (FACS) analysis using an anti-CD24 antibody, that a subset (~25%) of human sperm expresses the glycoprotein CD24, a known ligand for Siglec-10 (Fig. 6d).

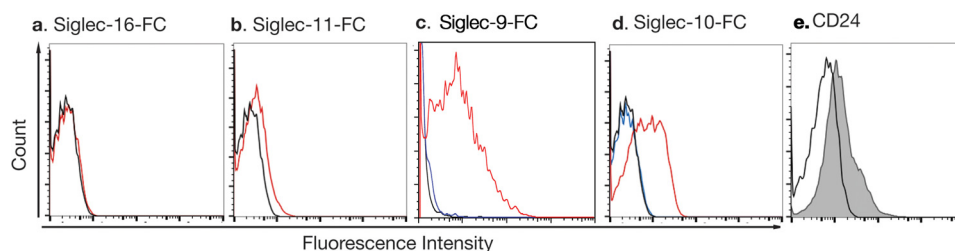


Figure 6. Human spermatozoa carry sialylated ligands for Siglec-10. Flow cytometry analysis indicates that human sperm do not bind Siglec-11-Fc (a) and Siglec-16-Fc (b) (black line is control and red is the Siglec-Fc fusion protein). Human sperm do bind Siglec-9 (c) and Siglec-10-Fc (d) in a sialic acid-dependent manner (black is control, red is WT Siglec-10-Fc, blue is Siglec-10(R120K)-Fc). A subset of sperm also express CD24 (e), a known sialylated ligand of Siglec-10.

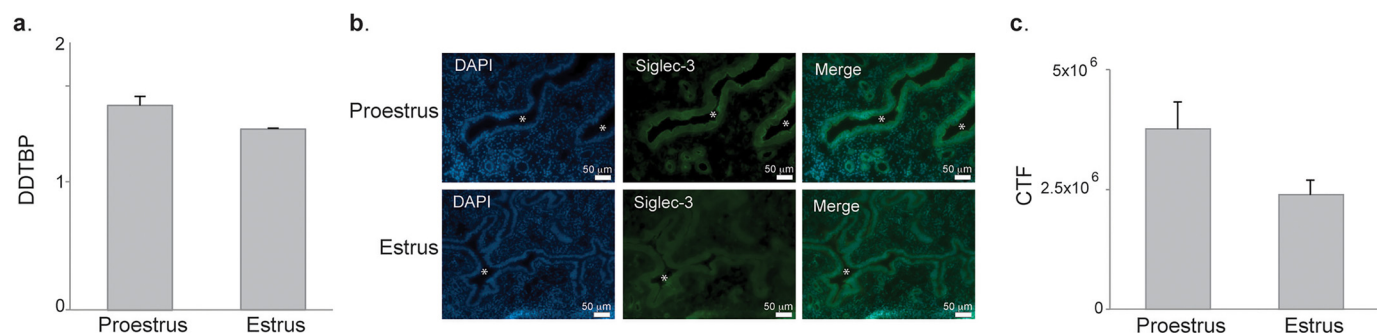


Figure 7. Siglec-3 is expressed in the mouse uterine epithelium. qPCR analysis (a) of the whole mouse uteri indicates that Siglec-3 expression is higher than Siglec-E and -F. Immunofluorescence staining for Siglec-3 (b) indicates Siglec-3 expression is enriched in the epithelium and epithelium glands as compared with the underlying stroma (asterisks indicate the uterine lumen). c, quantification of Siglec-3 antibody staining.

Mouse endometrium also expresses Siglecs

We initially focused on Siglec-G, the mouse homologue of Siglec-10 that was expressed in the human post-mortem endometrium and human endometrial cell lines. However, both IHC and FACS analyses revealed an absence of Siglec-G expression in the mouse endometrium. We then screened the expression of other Siglecs in mouse uterus by qPCR (Fig. 7a). We found that Siglec-3 was highly expressed in the mouse uterus and confirmed the presence of Siglec-3 protein by IHC analysis, specifically in the uterine epithelium (Fig. 7b).

Discussion

As sialic acids and Siglecs have well-established roles in modulation of immune responses, we hypothesized that sperm sialic acids could interact with various inhibitory Siglecs expressed by neutrophils to inhibit their activation during the leukocytic reaction. By using *in vitro* capacitation as a biologically relevant way of shedding sialic acids from sperm, we sought to determine whether capacitated sperm, with reduced levels of sialic acid, activated neutrophils more than noncapacitated sperm. Surprisingly, we determined that co-incubation of neutrophils with capacitated sperm did not result in additional neutrophil reactivity than when neutrophils were co-incubated with NC sperm. Moreover, co-incubation of neutrophils with C sperm resulted in similar levels of neutrophil reactivity when measured via CD11b levels, reactive oxygen species production, and in the shedding of L-selectin (36). Furthermore, neutrophils are capable of capturing both NC and C sperm via the formation of NETs *in vitro*. These data indicate that high levels of sperm-associated sialic acids do not reduce neutrophil activation *in vitro*.

Although sperm capacitated *in vitro* shed sialic acid, some residual sialic acid is retained on the sperm surface (19). This

residual sialic acid may be sufficient to engage inhibitory Siglecs on neutrophils, explaining the lack of differences in neutrophil activation as compared with NC sperm. In addition, the higher mortality of sperm kept under noncapacitating conditions *in vitro* may confound the interpretation of the data. Although efforts are made to keep the number of live sperm cells equal between conditions, NC sperm have a higher rate of cell death than C sperm, as evidenced by a higher proportion of propidium iodide-positive cells (Fig. S6). The high rate of death in NC sperm likely results in the presentation of danger-associated molecular patterns (DAMPs) that have been shown to contribute to the overall reactivity of nearby immune cells (37). Controlling for a higher level of dead or moribund sperm in the noncapacitated condition is challenging because viability-enhancing components such as albumin also function as key capacitation factors *in vitro*. Although future experiments might address neutrophil reactivity to increasing proportions of dead sperm, the current data must be interpreted with the caveat that the observed neutrophil reactivity toward NC and C sperm is confounded by a roughly 20% difference in sperm viability between the two conditions. In addition, others have previously suggested that a primary purpose of the leukocytic reaction is to remove dead and/or dying sperm (38). As the NC sperm samples contain a higher percentage of such cells, the resultant DAMPs may generate a danger signal that overpowers the reduction of immunoreactivity imparted by a sperm sialic acid-inhibitory Siglec interactions.

Aside from immune cells, sperm also come into contact with the endometrium during the leukocytic reaction. We sought to investigate if sperm could be interacting with the endometrium in a sialic acid-dependent manner. Unlike for neutrophils, the expression of Siglecs in the uterus has not previously been

Endometrial Siglecs

reported. We have now documented the expression of Siglecs-10, -11, and -16 in human endometrial cell lines for the first time. Siglecs-10 and -11 are inhibitory receptors and Siglec-16 is an activating receptor that is known to be polymorphic in humans with a common loss-of-function (pseudogenized) variant (44). Endometrial cells also express SHP1 and DAP12, the respective downstream signaling components of inhibitory and activating Siglecs. Preliminary bioinformatic analysis (using oPOSSUM) indicates that estrogen response elements are in the genomic sequences flanking Siglec-11 and Siglec-16. Transcription-binding sites for one of more transcription factors (AP-1, SP-1, NF- κ B, and CREB1) known to act in the tethering pathway for estrogen receptors have been identified in the genomic sequences flanking all three Siglecs by bioinformatics analysis. The presence of these transcription factor-binding sites indicates that Siglecs expressed by endometrial cells may be transcriptionally up-regulated by estrogen exposures. Future experiments where the expression of endometrial Siglecs before and after exogenous estrogen treatment should be very informative in this regard.

We have shown Siglec-10 expression in pre-menopausal human endometrium and have established that highly sialylated noncapacitated human sperm bind Siglec-10 in a sialic acid-dependent manner. We also report that a subset of sperm bears CD24, a sialic acid containing glycoprotein that is an established activator of Siglec-10 signaling (39). Although a recent paper has identified the presence of Siglec-10 in first trimester placental cells (40), the majority of work on Siglec-10, and its mouse homologue Siglec-G, concerns its role in the immune system. Generally, Siglec-10/G is considered an inhibitor of immune responses in various cellular contexts. For example, mouse Siglec-G functions in B1a cells to inhibit their proliferation and induce their tolerance to self-antigens (41). Consequently, genetic ablation of Siglec-G (in select genetic backgrounds) results in an age-dependent autoimmunity phenotype and increases the severity of collagen-induced arthritis in an autoimmune prone mouse model (42). CD24 is a ligand for Siglec-G and Siglec-10 in mouse and human, respectively. *In vivo*, Siglec-G and CD24 are required to inhibit the production of inflammatory cytokines in an acetaminophen-induced liver necrosis model and graft *versus* host disease (39). Our results indicate that sialic acid-containing ligands, including those carried by CD24, on human sperm may interact with endometrial expressed Siglec-10 and with neutrophil Siglec-9 during the leukocytic reaction (Fig. 8). *In vivo*, this interaction could inhibit the release of cytokines by the endometrium, decrease the recruitment and activation of leukocytes to the uterine lumen during the leukocytic reaction, and result in increased sperm survival. Future experiments exposing live sperm to cultured female tract cell lines should be very informative in this regard.

Although Siglec-11 and Siglec-16 were expressed by the endometrial cell lines, the Siglec-11-Fc and Siglec-16-Fc proteins did not bind to human sperm *in vitro*. Although Siglecs are defined as sialic acid-binding lectins, the precise specificities for sialylated glycan patterns of each Siglec remain poorly defined. In addition, Siglecs aggregate on the cell surface and such aggregation strongly influences their binding to *cis* or *trans* ligands (43). The use of soluble Siglec-Fc proteins as molecular

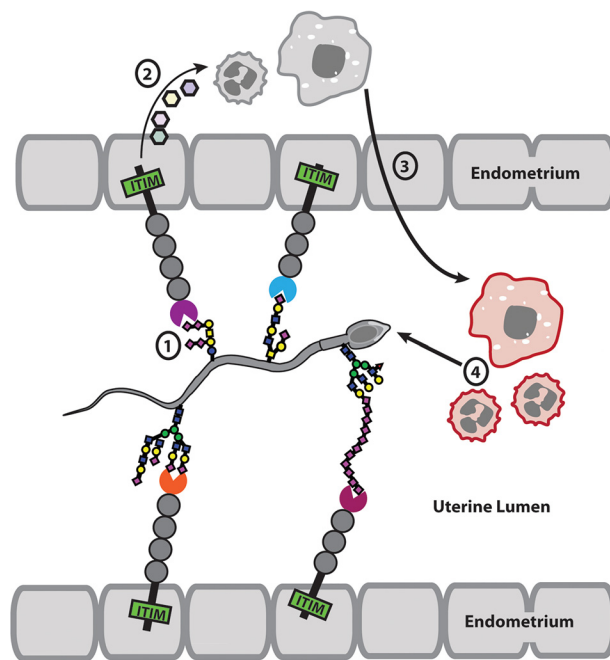


Figure 8. Sperm sialic acid and endometrial Siglecs may interact to modulate the female immune response to sperm. Both human and the mouse endometrium express inhibitory Siglecs (Siglec-10 and Siglec-3, respectively). When bound by sialic acid on sperm, the expressed inhibitory Siglecs may inhibit the immune response of the endometrium by inhibiting the release of cytokines, complement or other pro-inflammatory proteins via SHP-1 signaling. Sperm may further directly interact with neutrophils and macrophages and inhibit their immune response.

probes does not fully mimic these cell-surface mechanisms. It is possible that sperm do carry ligands for Siglec-11 and Siglec-16, but that using the Siglec-Fc fusion proteins does not allow to reconstitute the *in vivo* cell-surface mechanisms. As Siglecs-11 and -16 are paired receptors, and their precise specificity for glycan patterns is poorly defined (a problem for Siglecs in general), they could possibly also bind molecular patterns of pathogens, rather than SAMPs, explaining their lack of binding to sperm in our assay.

To generate a model for investigating the role of Siglec-10 in the leukocytic reaction, we sought to determine the expression of Siglec-G, the mouse homologue of Siglec-10, in the mouse uterus. We were surprised to find that Siglec-G was not expressed in the mouse uterus. We did, however, determine that another inhibitory Siglec, Siglec-3, is expressed in the mouse uterine epithelium and may be under hormonal control. Although no reproductive problems have been reported within Siglec-3 knockout mice, it would be very interesting to determine whether these female mutants show increased infiltration of leukocytes into the uterine lumen after mating.

In conclusion, we provide definite evidence for the presence of Siglec innate immune receptors in the female reproductive tract and their capacity to recognize sialylated glycans on sperm in humans and mice. Our results suggest that sperm engage female innate immune receptors *en route* to fertilization. The female immune system may spare some allogeneic sperm cells, in part because sperm are coated with highly sialylated glycan patterns that is shared between sperm and local female cells. Altogether, our results suggest that the immune paradox of mammalian insemination may

in part be resolved via the sensing of shared, species-specific glycan patterns on sperm by innate immune-modulating receptors expressed along the endometrium. Further insights into the precise mechanism of these interactions will require *in vitro* experiments with human sperm and endometrial cell lines to measure alterations in endometrial cell signaling cascades and/or cytokine expression.

Experimental procedures

In vitro interactions between human sperm and activated neutrophils

Human sperm were collected from volunteers via masturbation under UCSD human subjects protocol number 16027, liquefied at room temperature for 30 min, and washed with TYH media before being exposed to NC TYH media or capacitating conditions (C) TYH plus 5 mg/ml of BSA and 15 mM NaHCO₃ for 4 h at 37 °C and 5% CO₂, as described previously (45). Blood was collected in heparin-coated tubes from human donors using UCSD human subjects protocol number 170921X. This study abides by the Declaration of Helsinki for all human sample collection. Red blood cells were removed from whole blood using an Easysep magnetic separation kit (Stemcell Technologies) with anti-glycophorin A-conjugated magnetic beads. The remaining plasma and leukocytes were co-incubated for 30 min with NC sperm, C sperm, or sperm-free capacitation media (C buffer alone), fixed in 10% PFA on ice for 20 min, and stained with antibody staining: FITC mouse anti-CD62L IgG1 (Biolegend, 304802) or mouse IgG1, κ -isotype control (BD Biosciences, 349041), FITC rat anti-CD11b (Tonbo Biosciences, 70-0112-OWL-A11503) or rat IgG2b, κ -isotype control (BD Biosciences, 554688). Phagosomal ROS production was analyzed using Fc-OxyBURST Green Assay Reagent (ThermoFisher, F2902) for 15 min and then fixed. The stained sperm and leukocytes were submitted to flow cytometry on a BD FACSCalibur. The population was gated based on forward and side scatter to analyze a minimum of 10,000 neutrophils for each experiment. Data were analyzed using FlowJo. NET formation was visualized using the live cell-impermeable DNA stain SYTOX Green (ThermoFisher, S7020).

Sialidase activity and confirmation of capacitation

Swim-up sperm were incubated with 100 μ M 4-MU-NANA (Sigma, M8639) and exposed to an equal volume of either NC or C buffer. Control samples contained the NC or C buffer alone. Samples were read on a Molecular Devices SpectraMAX M3 plate reader using excitation and emission values of 365 and 450 nm, respectively. Confirmation of sperm capacitation was performed after 1 h of capacitation with the MitoProbe JC-1 Assay Kit for Flow Cytometry (Molecular Probes, MP34142) following the manufacturer's recommendations. Tyrosine phosphorylation was confirmed by staining with anti-phosphotyrosine mouse monoclonal at dilution of 1:10,000 (Cell Signaling, 9411).

Endometrial cell line maintenance and flow cytometry experiments

Ishikawa cells and HEC-1B cells were purchased from Sigma or ATTC, respectively. Both cell lines were maintained in RPMI

with phenol red and supplemented with 10% FBS, 1% penicillin/streptomycin, and 1% L-glutamate. For the flow cytometry based screen for Siglec expression, cells were fixed for 10 min in 10% PFA. 10⁶ cells were submitted to antibody staining for each experiment. The following primary antibodies were used: APC mouse anti-human Siglec-3 IgG1 (BD, 551378), PE mouse anti-human CD170 (Biolegend, catalog number 352004), APC mouse anti-human Siglec-7 IgG1 (R&D Systems, FAB1138), mouse anti-human Siglec-9 (R&D Systems, AF1139), APC mouse anti-human Siglec-10 (Biolegend, 347606), mouse antihuman Siglec-11 (R&D Systems, MAB3258), mouse anti-human Siglec-11 (R&D Systems, MAB3258), rabbit anti-human SHP-1 (Abcam, 131537), and goat anti-human DAP12 (Novus Biologicals, NBP1-52376). All unconjugated Siglec antibodies were stained with Alexa Fluor 647 goat anti-mouse IgG. The SHP-1 antibody was stained with Alexa Fluor 488 goat anti-rabbit IgG. The DAP12 antibody was stained with Alexa Fluor 647 donkey anti-goat IgG. Siglec staining was determined by flow cytometry on a BD FACSCalibur. A minimum of 10,000 events were counted for each experiment. Data were analyzed using FlowJo.

Siglec-Fc fusion protein purification

Purification of human Siglecs was performed as previously described (46). Briefly, 18 μ g of each human Siglec-Fc plasmid were transfected into 2 \times 10⁷ HEK293A cells. Transfected cells were grown in 50% Dulbecco's modified Eagle's medium, 50% RPMI supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, and 1% Nutridoma for 4 days. Secreted Siglec-Fc proteins were purified from conditioned media using protein A-Sepharose beads and treated with *Arthrobacter ureafaciens* sialidase (AUS) to remove any possible interaction with sialic acid containing secreted proteins. All Siglec-Fc encoding plasmids were kindly provided by Dr. Ajit Varki.

Human sperm isolation and binding to Siglec-Fc by flow cytometry analysis

Human ejaculate was donated by volunteers recruited under UCSD IRB protocol number 160274. To remove seminal proteins and any other contaminants, donated ejaculate was washed three times with 10 times volume of PBS and filtered through glass wool. The isolated sperm were then fixed in 2% PFA for 10 min at room temperature. 10⁶ sperm cells in suspension were incubated with a Siglec-Fc for 30 min followed by a 30-min incubation with R-phycoerythrin AffiniPure F(ab')₂ fragment goat anti-human IgG, Fc γ fragment specific (Jackson ImmunoResearch, 109-116-170). For CD24 staining, 10⁶ sperm cells were incubated with Alexa Fluor 647 mouse anti-human CD24 (Biolegend, 311109). Binding of the Siglec-Fc and CD24 expression was determined by flow cytometry on a BD FACSCalibur. A minimum of 10,000 events were counted per experiment. Data were analyzed using FlowJo.

Mouse estrus tracking and uterine qPCR

C57/B6 mice were kept under UCSD IACUC protocol number S16223. The estrus cycle was tracked by vaginal cytology for a minimum of 2 weeks before mice were sacrificed. Briefly, the vagina of mice was washed with \sim 100 μ l of PBS and the isolated

Endometrial Siglecs

cells were analyzed under a dissecting microscope. The stage in the estrus cycle was determined by the proportion of cornified epithelial cells, nucleated epithelial cells, and leukocytes as described in Caligioni (47). 3 to 4-month-old female in proestrus or estrus were humanely euthanized. Harvested uterine horns were homogenized. Total mRNA was isolated using the Qiagen RNA mini kit, which contains on column DNase. All isolated RNA was submitted to UV spectroscopy and samples had A_{260}/A_{280} readings between 1.8 and 2.0. For all samples 2 μg of RNA were converted to cDNA using the Bio-Rad iScript kit. qPCR was performed using gene-specific primers, the Power SYBR Green master mix (Invitrogen, number 4367659), and the Bio-Rad CFX96 Real-time system. Sequence for Siglec-3 primers (ID: 10946590a1), Siglec-E (ID: 13626036a1), and Siglec-F (ID: 28864163a1) used were taken from Primerbank (48). qPCR was performed on two biological replicates in triplicate. C_t values were normalized to the C_t values for TBP. TBP was used as a reference gene as its C_t values were more similar to that the Siglecs.

Immunofluorescence staining of human and mouse uterus

Frozen samples of pre-menopausal human uterine biopsies were kindly provided by Dr. Nissi Varki. Mouse uterine horns at pro-estrus and estrus were harvested from 3- to 4-month-old mice. Both human and mouse tissues were flash frozen in optimal cutting temperature (OCT[®]) and sectioned into 7- μm slices. Human endometrial samples were stained with goat anti-human Siglec-10 (Santa Cruz, sc-240882) or mouse anti-human Siglec-11 (R&D Systems, MAB3258) and visualized with Cy3 bovine anti-goat IgG (Jackson ImmunoResearch, 705-165147) or Cy3 donkey anti-mouse IgG (Jackson ImmunoResearch, 715-165-150), respectively. Mouse uteri were stained with rabbit anti-mouse Siglec-3 (Santa Cruz, sc-28810) and visualized with Alexa Fluor 488 goat anti-rabbit IgG (A11008). All images were taken using Zeiss AXIO Observer D1 Inverted Disc Fluorescence Microscope. For image quantification, exposure time was the same and the fluorescence intensity for a given antibody stain was quantified using ImageJ. Five images from four different mice were used for quantification.

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