Title

Host and pathogen hyaluronan signal through human siglec-9 to suppress neutrophil activation

Journal

Journal of Molecular Medicine, 94(2)

ISSN

0946-2716

Authors

Secundino, Ismael

Lizcano, Anel

Roupé, K Markus

et al.

Publication Date

2016-02-01

DOI

10.1007/s00109-015-1341-8

Peer reviewed

HHS Public Access

Author manuscript

J Mol Med (Berl). Author manuscript; available in PMC 2017 February 01.

Published in final edited form as:

J Mol Med (Berl). 2016 February ; 94(2): 219–233. doi:10.1007/s00109-015-1341-8.

Host and Pathogen Hyaluronan Signal Through Human Siglec-9 to Suppress Neutrophil Activation

Ismael Secundino[1,2,3], **Anel Lizcano**[1,4], **K. Markus Roupé**[1,2,5], **Xiaoxia Wang**[1,4], **Jason N. Cole**[2,6], **Joshua Olson**[2], **S. Raza Ali**[1,2], **Samira Dahesh**[2], **Lenah K. Amayreh**[2], **Anna Henningham**[1,2], **Ajit Varki**[1,4,7], and **Victor Nizet**[1,2,8]

Ajit Varki: a1varki@ucsd.edu; Victor Nizet: vnizet@ucsd.edu

[1]Glycobiology Research and Training Center, UC San Diego, La Jolla, California, United States of America

[2]Department of Pediatrics, UC San Diego, La Jolla, California, United States of America

[3]Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, México

[4]Department of Cellular and Molecular Medicine, UC San Diego, La Jolla, California, United States of America

[5]Faculty of Medicine, Lund University, Lund, Sweden

[6]Australian Infectious Diseases Research Centre, University of Queensland, St. Lucia, Queensland, Australia

[7]Department of Medicine, UC San Diego, La Jolla, California, United States of America

[8]Skaggs School of Pharmacy and Pharmaceutical Sciences, UC San Diego, La Jolla, California, United States of America

Abstract

Inhibitory CD33-related Siglec receptors regulate immune cell activation upon engaging ubiquitous sialic acids (Sias) on host cell surface glycans. Through molecular mimicry, Sia-expressing pathogen group B *Streptococcus* binds inhibitory human Siglec-9 (hSiglec-9) to blunt neutrophil activation and promote bacterial survival. We unexpectedly discovered that hSiglec-9 also specifically binds high molecular weight hyaluronan (HMW-HA), another ubiquitous host glycan, through a region of its terminal Ig-like V-set domain distinct from the Sia-binding site. HMW-HA recognition by hSiglec-9 limited neutrophil extracellular trap (NET) formation, oxidative burst, and apoptosis, defining HMW-HA as a regulator of neutrophil activation. However, the pathogen group A *Streptococcus* (GAS) expresses a HMW-HA capsule that engages hSiglec-9, blocking NET formation and oxidative burst, thereby promoting bacterial survival. Thus, a single inhibitory lectin receptor detects two distinct glycan “self-associated molecular

Anel Lizcano, K. Markus Roupé, Xiaoxia Wang and Jason N. Cole contributed equally the work.

Conflict of Interest

The authors declare no competing financial interests.

patterns" to maintain neutrophil homeostasis, and two leading human bacterial pathogens have independently evolved molecular mimicry to exploit this immunoregulatory mechanism.

Keywords

CD33-related Siglecs; Hyaluronan; neutrophils; Group A *Streptococcus* (GAS)

Introduction

Siglecs (Sialic acid-binding Ig-like lectins) are type I transmembrane proteins with an N-terminal immunoglobulin (Ig)-like-V-set domain mediating sialic acid (Sia) recognition, followed by a variable number of Ig-like-C-2 set domains, a transmembrane domain, and often a cytoplasmic tail with one or more immunoreceptor tyrosine-based inhibitory motif (ITIM) that recruits tyrosine phosphatases such as SHP-1 [1]. Inhibitory CD33-related Siglecs are mammalian immune cell receptors that dampen cell activation upon engagement of sialylated-terminated glycans prominent on cell surface glycoproteins and glycolipids [2, 3]. Among this rapidly evolving gene family, nine inhibitory CD33-related Siglecs have been characterized in humans (hCD33, hSiglec-5 to hSiglec-12), whereas mice have only four (mCD33, mSiglecE-G) [1, 2, 4]

Sialic acids can function as ubiquitous self-associated molecular patterns (SAMPs) [5] recognized by these inhibitory CD33-related Siglecs (CD33rSiglecs) to maintain the baseline non-activated state of innate immune cells [6, 7]. This self-recognition helps to counter-regulate inflammatory responses activated upon sensing of damage-associated molecular patterns (DAMPs) [8] including high mobility group box-1 (HMGB1) [9], ATP [10], heat shock proteins [11] and mitochondrial DNA [12] or pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), peptidoglycan and flagellin [13].

If regulation by inhibitory CD33rSiglecs is perturbed, pathologies may ensue including eosinophilic airway inflammation in mSiglec-F knockout mice [14], elevated pro-inflammatory cytokines in mice lacking mSiglec-G [8], asthma associated with hSiglec-8 polymorphisms [15], or exaggerated T-cell responses linked to an hSiglec-9 gene polymorphism [16]. Mouse microglial cells lacking mSiglec-E showed increased inflammatory responses and neurotoxicity in neuronal co-culture experiments [17], and hSiglec-10 is a selective modulator of the immune response to the DAMP HMGB1 released by necrotic cells [8]. These lines of investigation identify CD33rSiglecs as important immune response regulators [7].

Neutrophils are 50%–70% of circulating leukocytes and represent a critical first line innate host defense mechanism [18]. Neutrophils migrate from the circulation to foci of infection in response to bacterial or host-derived chemoattractants, inflammatory cytokines and endothelial adhesion molecules. Neutrophils carry out microbicidal activities including phagocytosis, generation of reactive oxygen species (ROS), degranulation to release antimicrobial peptides and proteases, and deployment of neutrophil extracellular traps (NETs) [18]. However, neutrophilic inflammation risks damaging host cells, and

homeostatic mechanisms are in place to allow resolution of the inflammatory response [18]. Inhibitory CD33-related hSiglec-9 is constitutively expressed by human neutrophils [19, 20] while mouse neutrophils possess inhibitory mSiglec-E [4]. We previously found that the human bacterial pathogen group B *Streptococcus* (GBS) uses Sia mimicry in its surface polysaccharide capsule to engage hSiglec-9 and mSiglecE, suppressing neutrophil activation [21, 22].

Like Sias, glycosaminoglycans (GAGs) are natural glycans highly enriched and widely distributed on vertebrate cells and extracellular matrices, but rarely expressed by microorganisms [23]. Hyaluronan (HA) is a GAG composed of repeating disaccharide units of N-acetyl-D-glucosamine (GlcNAc) and D-glucuronic acid (GlcA) with alternating β1–4 and β1–3 linkages i.e., (GlcNAcβ1–4GlcAβ1–3)n typically existing in a native high molecular weight of >1,000 kDa state (HMW-HA) [24]. HMW-HA is abundant in many tissues including synovium [25], heart valves [26], skeletal tissues [27] and skin [28], where its functions include space filling, hydration/lubrication, and provision of a matrix through which cells can migrate [29].

HA exerts different biological activities depending on its molecular mass; whereas low molecular weight HA fragments (LMW-HA), released under inflammation and tissue injury conditions, tend to induce inflammation by inducing pro-inflammatory cytokines and chemokines (TNF-α, IL-1β, IL-8, MIP-lα/β, RANTES, MCP-1), promoting cell proliferation and angiogenesis [30, 31]. In contrast, native HMW-HA may act to mitigate inflammatory damage by downregulating the inflammatory response and HA turnover [24, 30, 32]. One mechanism by which HA modulates the inflammatory response is through recognition by cell-surface glycoprotein CD44 [33]. CD44-deficient mice exhibit exaggerated TLR4-mediated sepsis responses to LPS [34], and had problems resolving inflammation as shown by impaired clearance of apoptotic neutrophils and persistent accumulation of LMW-HA at the site of tissue injury [35].

Here, when GAGs were used as a “control” for negatively charged glycans in studying human CD33rSiglec interactions, we unexpectedly observed a strong selective recognition of HMW-HA by human Siglec-9 on neutrophils. This interaction increased SHP-1 recruitment to the inhibitory receptor and suppressed neutrophil oxidative burst, NET formation, and apoptosis. Domain mapping identified the first Ig-like domain as responsible for HMW-HA binding, independent of Sia binding. We found that this dynamic interaction was exploited by the human-specific bacterial pathogen, group A *Streptococcus* (GAS), which uses molecular mimicry in its HMW-HA capsule to suppress neutrophil activation and promote its own survival. A weaker binding interaction of HMW-HA to inhibitory mSiglec-E on mouse neutrophils allowed us to further corroborate the functional impact of this discovery using WT and KO animals *ex vivo* and *in vivo*.

Materials and methods

Ethics Statement

Simple phlebotomy for neutrophil collection from healthy adult donors was performed with informed consent under a protocol approved by the University of California San Diego

(UCSD) Human Research Protection Program. Animal studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health under a protocol approved by the UCSD Institutional Animal Care and Use Committee at the University of California, San Diego. All efforts were made to minimize suffering of animals employed in this study.

Siglec-Fc Purification

Siglec-9-EK-Fc/pcDNA3.1 construct expressed a recombinant soluble human Siglec-9–Fc protein (hSiglec-9–Fc) with three extracellular Ig-like domains of hSiglec-9 attached to human IgG Fc. For hSiglec-5/7 and −11–Fc, we used constructs encompassing 4, 3 and 5 extracellular Ig-like domains, respectively. The hSiglec-9–Fc^{R120K} contains Arg →Ala mutation in the V-set domain, known to eliminate sialic acid recognition [19]. Fusion proteins were prepared by transient transfection of Chinese hamster ovary TAg cells following the established protocol [19]. Siglec-Fc proteins were purified from culture supernatant by adsorption to protein A-Sepharose (GE Healthcare); human CD44–Fc chimera was purchased from R&D Systems. hSiglecs-Fc and CD44–Fc were treated with 25 mU and 5 mU of *Arthrobacter ureafaciens* sialidase (AUS) for 60 min to remove Sias, prior to elution from the protein A-Sepharose.

Preparation of Multiple Siglec-9–Fc Expression Fusion Constructs

A DNA fragment of human Siglec-9 encoding the first two Ig-like domains was amplified by Pfu-Ultra high-fidelity polymerase (Stratagene) following their protocol using 5’-AAGCTTCAGACAAGTAAACTGCTGACG-3’ (HindIII site) + 5’-TCTAGAGCCGTCTCCTTGGAAGAC-3’ (XbaI site) as primers and human Siglec9EK–Fc-pEDdC as template. The amplicon was cloned into expression vector Signal pIgplus MCSvector (Lab Storage), giving rise to a fusion protein of Siglec-9 extracellular domains and a human IgG Fc tail (hSiglec-9–Fc 2D). A fusion Fc construct encoding the first Ig-like domain (V-set) of hSiglec-9 and the second Ig-like domain (C2-set) of hSiglec-7 was made through two-step cloning. hSiglec-9 V-set domain was amplified using 5’-AAGCTTCAGACAAGTAAACTGCTGACG-3’ (HindIII site) + 5’-TCTAGATGTCACATTCACAGAGAGCCG-3’ (XbaI site) as primers and human Siglec9EK-Fc-pEDdC as a template. The fragment was cloned into Signal pIgplus MCSvector. The positive clone containing the inserted human Siglec-9 V-set domain was used as the vector for next-step cloning. hSiglec-7 C2-set domain was amplified using primers 5’-TCTAGAGCCTTGACCCACAGGCCCAAC-3’ (XbaI site) + 5’-GGATCCGTGCCTTCTCCTTGGAAGAC-3’ (BamHI site) and Siglec7EK-Fc-pEdDC as template. The fragment was cloned into Signal pIgplus MCSvector carrying human Siglec-9 V-set, giving rise to a fusion protein of hSiglec-9 V-set domain, hSiglec-7 C2-set domain and a human IgG Fc tail (hSiglec-9V-7C2–Fc). A fusion Fc construct encoding the first Ig-like domain (V-set) of hSiglec-7 and the second Ig-like domain (C2-set) of hSiglec-9 was also made through two-step cloning. Siglec-7 V-set domain was amplified using primers 5’ -AAGCTTCAGAAGAGTAACCGGAAGGAT-3‘ (HindIII site) and 5’ -TCTAGATGTCACGTTCACAGAGAGCTG-3 ‘ (XbaI site underlined) and hSiglec7EK-Fc-pEDdC as template. The fragment was cloned into Signal pIgplus MCSvector. The positive clone containing the inserted hSiglec-7 V-set domain was used as the vector for the

next step cloning. hSiglec-9 C2-set domain was amplified using 5' - TCTAGAGCCTTGACCCACAGGCCCAAC-3 ' (XbaI site) and 5' -- GGATCCGTGCCGTCTCCTTGGAAGAC-3 ' (BamHI site) as primers and Siglec9EK-Fc-pEdDC as template. The fragment was cloned into Signal pIgplus MCSvector carrying hSiglec-7 V-set, giving rise to a fusion protein of hSiglec-7 V-set domain, hSiglec-9 C2-set domain and a human IgG Fc tail (hSiglec-7V-9C2–Fc). T4 DNA ligase (Invitrogen) was used for the DNA ligation. DNA sequencing verified the coding sequences remained in-frame. The Fc chimera proteins were prepared by transient transfection of Chinese hamster ovary TAg cells with Siglec-Fc constructs following the established protocol [19]. Siglec-Fc proteins were purified from culture supernatant by adsorption to protein A-Sepharose, hSiglecs-Fc chimeric proteins were treated with 25 mU AUS for 60 min at room temperature to remove Sia. Acquired Fc proteins were quantified using the BCA protein assay reagent (Pierce).

Recognition of High Molecular Weight Hyaluronan (HMW-HA) by hSiglec-9–Fc

The binding of hSiglec-9/5/7/11 and hCD44–Fc chimeras to HMW-HA (Sigma-Aldrich) was determined using a previously described method with minor modifications. Briefly, 10 μg/well HMW-HA was covalently bound to CovaLink plates (Thermo Scientific) using 1% EDC (1-ethyl-3[3-dimethylaminopropyl]carbodiimide hydrochloride) (Thermo Scientific). Plates were incubated for 2 h at 37°C and then overnight at room temperature. Wells were blocked with 1% BSA/PBS for 2 h at room temperature. hSiglec-9–Fc was diluted in 20 mM Tris (pH = 8.0), 150 mM NaCl, 1% BSA at 0.125 μg/well and incubated for 2 h at 37°C. Anti-human IgG-HRP (Biorad) was used as secondary antibody at 1:5,000 dilution and incubated for 1 h at 37°C. TMB substrate solution (BD Biosciences) was added and the absorbance was detected at 450 nm.

Specific Inhibition of Binding of Siglec-9 to HMW-HA

The glycosaminoglycans (GAGs) used in this study included HMW-HA from rooster comb or bovine vitreous humor, HMW-heparan sulfate (HS) from bovine kidney, chondroitin sulfate A (CSA) from bovine trachea, heparin from bovine lung (all purchased from Sigma-Aldrich), and differentially sized HA fragments from Hyalose. HMW-HA was immobilized to CovaLink plates as described above. hSiglec-9–Fc (0.125 μg/well) was pre-incubated for 30 min at 37°C in the presence of GAGs or HA fragments in binding buffer: 20 mM Tris (pH=8.0), 150 mM NaCl, 1% BSA). Then, hSiglec-9–Fc/GAGs were added to wells and incubated for 2 h at 37°C. Anti-human IgG-HRP (Biorad) was used as secondary antibody at 1:5,000 and incubated for 1 h at 37°C. Absorbance was detected at 450 nm.

Assay for Siglec-Fc Binding to Bacteria

The interaction of hSiglec-9 with bacteria was determined using a previously described method [36] with minor modifications. Immulon ELISA plates were coated with 0.025 mg/ml protein A (Sigma-Aldrich) in coating buffer (67 mM $NaHCO_3$, 33 mM Na_2CO_3, pH=9.6) overnight at 4°C. Wells were washed and blocked with assay buffer (20 mM Tris pH=8.0, 150 mM NaCl, 1% BSA) for 1.5 h at 37°C. Aliquots of hSiglec-9–Fc diluted in assay buffer were added to individual wells at 0.025 mg/ml for 2 h at 37°C. GAS strains were labeled with 0.1% FITC (Sigma) for 1 h 37°C and then suspended at 1×10^7 cfu/ml in

assay buffer, then strains were added to each well and centrifuged at 805 × *g* for 10 min. For competition assays, before adding bacterial strains, HMW-HA from rooster comb or bovine vitreous humor (Sigma-Aldrich), heparin from bovine lung (Sigma-Aldrich) and heparan sulfate purified from CHO cells were added to the wells and incubated for 60 min. Bacteria were allowed to adhere for 15 min at 37°C, wells were washed to remove unbound bacteria, and the residual fluorescence intensity (excitation, 485 nm; emission, 538 nm) measured using a Spectra Max Gemini XS fluorescence plate reader (Molecular Devices).

Blocking Antibodies

Human neutrophils were purified from normal human volunteers using the PolyMorphPrep system (Axis-Shield), suspended in RPMI 1640 medium + 2% autologous heat-inactivated human plasma. Two commercial anti-hSiglec-9 antibodies were assessed for their ability to block the binding of HMW-HA to human neutrophils: mouse monoclonal anti-hCDw329 (BD Biosciences Pharmingen, #550906) and goat anti-hSiglec-9, (R&D Systems #BAF1139). For blocking binding of HMW-HA to CD44, mouse anti-human CD44 (Thermo Scientific #MS-178-PABX) was used. Fluorescein-HA (Sigma-Aldrich) at 10 μg/ml was incubated for 60 min at 4°C and binding measured by FACS.

Neutrophil adhesion to immobilized HMW-HA

5×10^5 neutrophils were labeled with FilmTracer Calcein green (Invitrogen) vital staining according to the manufacturer instructions. 1 μg/ml HMW-HA (Sigma) was bound to 96-well plate and blocked with 3% BSA, then neutrophils plated and incubated at 37°C for 30 min. After washing the plate with 1% BSA in Hanks' balanced salt solution (HBSS), adherent cells were visualized under a fluorescent microscope and enumerated by counting in a hemocytometer. As a control for specific binding to HMW-HA and hSiglec-9, neutrophils were incubated α-Sig-9(HA) to inhibit hSiglec-9:HA interaction, α-Sig-9(Sia) to inhibit hSiglec-9:Sia recognition or α-CD44 Abs for 10 min.

SHP-1 Recruitment

Human neutrophils were seeded into 6-well plates at 1.2×10^7 cells and pretreated in the presence/absence HMW-HA and ± 25 nM of phorbol −12myristate 13-acetate (PMA). Protein concentration was normalized to 1 mg and immunoprecipitation performed using goat anti-Siglec-9, (R&D Systems #BAF1139) at 2 μg/ml in the presence of 5x protease inhibitor cocktail, phosphatase inhibitors (50 mM Na_3O_4V,10 mM NaF, 20 mM imidazole, 5 mM Na Molybdate) and 5 mU micrococcal nuclease for 12 h at 4°C. The next day, protein-G Sepharose beads were added for 3 h at 4°C. Proteins were separed by reducing SDS-PAGE, transferred to PVDF and probed with anti-Siglec-9 (R&D Systems #BAF1139) and rabbit anti-SHP-1 (Santa Cruz Biotechnology #sc-287) an appropriate HRP-conjugated secondary antibody and quimioluminicence substrate (Thermo Scientific).

Oxidative Burst Assay

2×10^6 neutrophils/ml were suspended in HBSS (Thermo Scientific) with Ca^{2+} and Mg^{2+} + 5.5 mM glucose in the presence of 10 μg/ml OxyBURST Green H_2HFF BSA (Molecular Probes) for 30 min. 5×10^5 PMNs were seeded into 24-well plates and pretreated in the

presence/absence of α-Sig-9(HA), α-Sig-9(Sia) or α-CD44 Abs at 3.2 μg/1×10^6 cells for 10 min, and then washed with 1% BSA in HBSS before use. Neutrophils were incubated with 10 μg/ml of HMW-HA or infected with GAS strains at a MOI (multiplicity of infection) = 20 bacteria per cell, and plates then centrifuged at 805 × *g* and incubated at 37°C, 5% CO_2 for 30 min. 25 nM of PMA was used as positive control. Neutrophils were gated according SSC/FSC and oxidative burst measured by FACS.

Neutrophil Extracellular Trap (NET) Assays

Neutrophils were seeded into 24-well plates at 5 × 10^5 cells/well and pretreated in the presence/absence of α-Sig-9(HA), α-Sig-9(Sia) or α-CD44 Abs at 3.2 μg/1×10^6 cells for 10 min and then washed with 1% BSA in HBSS before use. PMNs were incubated with 10 μg/ml of HMW-HA or infected with GAS strains at a MOI (multiplicity of infection) = 10 bacteria per cell, and then incubated with 25 nM of PMA in RPMI + 2% heat-inactivated FBS (66°C) at 37°C, 5% CO_2 for 3 h to induce NET release. Cells were fixed with 4% paraformaldehyde for overnight at 4°C, and then cells were washed with PBS and blocked with 2% BSA-PBS + 2% goat serum for 45 min at room temperature. Cells were stained with rabbit anti-human myeloperoxidase (Dako) at 1:300 for 1 h at room temperature. Neutrophils were washed and visualized by incubation with secondary antibody AlexaFluor488 goat anti-rabbit IgG (Invitrogen) and DAPI at 1:500 and 1:10,000, respectively for 45 min at room temperature in the dark. NETs were visualized under a fluorescent microscope. To quantify NET DNA release, 500 mU micrococcal nuclease was added for 10 min at 37°C and the reaction stopped with 5 mM EDTA. The plate was centrifuged at 200 × *g* for 8 min and the supernatant was transferred into a 96-well plate, mixed with 100 μl of Quanti-iT Picogreen (Invitrogen), and incubated for 2–5 min at room temperature in the dark. Fluorescence intensity (excitation, 485 nm; emission, 538 nm) was measured using a Spectra Max Gemini XS fluorescence plate reader (Molecular Devices). Concentration of extracellular DNA was expressed as percentage of DNA from experimental wells compared to total DNA of 5 × 10^5 cells.

Terminal Deoxynucleotidyl Tranferase dUTP Nick End Labeling (TUNEL) Assay

Neutrophils were seeded into 24-well plates at 2 × 10^6 cells/well, pretreated in the presence/absence of α-Sig-9(HA) or isotype control at 3.2 μg/1×10^6 cells for 10 min, then washed with 1% BSA in HBSS before use. Neutrophils were incubated with 10 μg/ml of HMW-HA. To avoid the effect of growth factors present in FBS, neutrophils were incubated in RPMI medium alone for 18 h. As a positive control, 5 μg of staurosporine was used. The % apoptosis was determined by dividing TUNEL-positive cells versus the total number of cells × 100.

Group A *Streptococcus* (GAS) Strains and Growth Conditions

Human GAS serotype M1T1 isolate 5448 was isolated from a patient with necrotizing fasciitis and toxic shock [37]. Its mouse-passaged derivative 5448 AP is a highly encapsulated strain [38]. The isogenic mutants deficient in synthesis of hyaluronan 5548 Δ*has A* [39] and Δ*sda1* [40] were described previously. All strains were propagated in Todd-Hewitt Broth (THB) to early log phase (OD_{600} of 0.4), collected and washed prior to assay.

Group B *Streptococcus* (GBS) WT strain serotype III (COH1), a heavily encapsulated isolate from a neonate with early-onset sepsis [41], was grown in THB to early log phase, collected and washed prior to assay.

Neutrophil Microbicidal Assays

Human neutrophils were suspended in RPMI 1640 medium + 2% autologous heat-inactivated human plasma and seeded in 96-well plates at 2×10^5 cells/well. Before infection, neutrophils were incubated with α-Sig-9(HA), α-Sig-9(Sia) or α-CD44 Abs at 3.6 μg/ml × 10 min then washed with 1% BSA in HBSS before use. Early logarithmic phase GAS (OD_{600} = 0.4), were suspended in RPMI 1640 medium + 2% heat-inactivated human plasma and added to neutrophils at MOI = 10 bacteria per cell. Plates were centrifuged at 805 × *g* and incubated at 37°C in 5% CO_2. After incubation for 30 min, neutrophils were lysed in dH_2O, serially diluted and plated on THA to enumerate surviving GAS colony forming units (CFU). Internal control wells without neutrophils were used to determine baseline bacterial counts at the assay endpoint. GAS % survival was calculated as (CFU/ml experimental well)/(CFU/control well) × 100.

Murine Siglec-E and Mouse Infection Studies

Binding of mSiglec-E and hSiglecs9/7 to HMW-HA was determined using an ELISA. Biotinylated HMW-HA was immobilized in microtiter wells with streptavidin, blocked and washed with 0.05% Tween-20/PBS, the respective Siglec-Fc chimeras added at 10 μg/ml for 2 h at room temperature, and binding detected with goat anti-human IgG-HRP and TMB substrate solution (BD Biosciences) at OD 400nm. Peritoneal neutrophils from WT and mSiglec-E KO mice were collected after 3% thioglycollate challenge, infected with log-phase WT GAS at MOI = 1 bacteria/neutrophil, and surviving bacterial CFU enumerated at 30 and 90 min after 0.025% Triton X100 lysis of cells. For blood survival, heparinized blood was collected by heart puncture from WT and mSiglec-E KO mice and 1×10^4 logarithmic phase WT or acapsular (Δ*hasA*) mutant bacteria added to 200 μl blood and surviving CFU enumerated at the indicated time points. In the *in vivo* infection, WT and mSiglec-E KO mice were infected with 1.0×10^7 CFU of logarithmic phase WT GAS in volume of 200 μl by peritoneal (IP) injection. Animals were euthanized 5 h post infection, and peritoneal fluid, liver and spleen collected for enumeration of CFU on THA after overnight incubation.

Results

Specific Recognition of Hyaluronan by the Ig-like-V-set Domain of hSiglec-9

When GAGs were used as a control in studying human CD33rSiglec interactions, we unexpectedly observed a strong selective recognition of HMW-HA by a hSiglec-9–Fc chimera (hSiglec-9–Fc) (Fig. 1a). HMW-HA was recognized by hSiglec-5–Fc to a much lesser extent, and did not interact significantly with hSiglec-7–Fc or hSiglec-11–Fc (Fig. 1a). Remarkably, hSiglec-9–Fc bound 3.4-fold better than CD44–Fc a well known HA receptor [33] (Fig. 1a).

To confirm selective recognition of HA by hSiglec-9, we performed competition assays using other mammalian GAGs with similar underlying backbone structures. While 10 μg/ml of soluble HMW-HA (GlcNAcβ1–4GlcAβ1–3)*n* blocked binding of hSiglec-9–Fc to immobilized HMW-HA, identical concentrations of HMW sulfated GAGs heparan sulfate and heparin with similar underlying backbones but more negative charge (GlcNAcα1–4GlcAβ1–4/IdoAα1–4)*n* or chondroitin sulfate with a partially shared disaccharide backbone and identical linkages (GalNAcβ1–4GlcAβ1–3)*n* did not interfere with hSiglec-9–Fc binding to HMW-HA (Fig. 1b).

The extracellular domain of hSiglec-9 has three Ig-like domains: an N-terminal V-set domain for Sia recognition followed by two C-2 set domains [19, 20]. To further map the domain on Siglec-9 responsible for HMW-HA recognition, we constructed different hSiglec-9 chimeric proteins in which the V-set domain or C-2 set domains were eliminated. To ensure correct folding, these hSiglec-9 Ig-like domains were expressed with either the V-set or C-2 set domain of hSiglec–7, a Siglec that did not bind to HMW-HA (Fig. 1a). Prominent HMW-HA binding was observed only in chimeric proteins in which the V-set domain of hSiglec–9 was present (e.g. Sig-9V-7C2), with reduced HMW-HA binding by hSiglec-9–Fc 2D (Sig-9 2D, lacking the third Ig domain), suggesting this Ig domain was critical for protein folding (Fig. 1c and 1d). In contrast, a chimeric protein containing the V-set domain of hSiglec–7 and the second C-2 set domain of hSiglec-9 (Sig-7V-9C2) did not bind to HMW-HA (Fig. 1c and 1d). As the V-set domain of hSiglec-9 was responsible for HMW-HA recognition, we evaluated binding of an hSiglec-9–Fc with an Arg→Ala mutation known to completely eliminate Sia recognition engineered in the V-set domain (hSiglec-9^{R120K}–Fc) [19], and saw only a modest decrease in binding (Fig 1c and 1d), indicating that the V-set domain of hSiglec-9 is responsible for both HMW-HA and Sia recognition, but through independent sites.

High Molecular Weight Hyaluronan (HMW-HA) is Recognized by hSiglec-9 on Human Neutrophils

HA exerts different biological activities depending on its molecular mass; whereas low molecular weight HA fragments (LMW-HA) tend to induce inflammation and cell proliferation, HMW-HA may act to mitigate inflammatory damage [30]. Competitions with soluble HMW-HA (>1,000 kDa) completely abrogated HA binding to hSiglec-9–Fc, whereas only partial inhibition was observed with soluble LMW-HA of 200 – 300 kDa or 25 – 75 kDa mass, and no inhibition using soluble oligo-HA (hexamer) and nano-HA (nonamer) fragments (Fig. 2a).

Inhibitory CD33-related Siglec-9 is prominently expressed on human neutrophils [19], and prior literature suggested that CD44 is the principal cellular surface counter-receptor involved in HA recognition [33]. We confirmed by flow cytometry that both hSiglec-9 and CD44 are constitutively expressed on our purified human neutrophils, with CD44 perhaps in higher abundance (Fig. 2b). FITC-labeled HMW-HA bound effectively to human neutrophils expressing the two receptors (Fig. 2c). To identify a tool for studying the role of hSiglec-9 in neutrophil-HA interactions, we evaluated two commercial antibodies (Abs) against hSiglec-9 – a mouse monoclonal Ab (mAb) manufactured by BD Pharmingen

(#550906) and a polyclonal Ab manufactured by R&D Systems (#BAF1139). Pre-exposure of human neutrophils to the former mAb inhibited their binding to FITC-labeled HMW-HA, whereas the latter Ab had no effect (Fig. 2d). Opposite results where observed using binding of hSiglec-9–Fc to the Sia-expressing capsule of GBS, wherein the #BAF1139 Ab blocked the interaction and the #550906 mAb had no effect (Fig. 5b). We designated the mAb that blocked the HMW-HA:hSiglec-9 interaction "α-Sig-9(HA)" and Ab that blocked the Sia:hSiglec-9 interaction "α-Sig-9(Sia)" for the analyses below.

HA plays a role in neutrophil recruitment [42], and we confirmed that human neutrophils bound to immobilized HMW-HA but not to an uncoated well (Fig. 2e and 2f). This interaction of neutrophils with immobilized HMW-HA was inhibited by α-Sig-9(HA) and an anti-CD44 blocking mAb (Thermo Scientific), but not by α-Sig-9(Sia) (Fig. 2e and 2f). Thus, hSiglec-9 and CD44 each contribute to neutrophil HMW-HA binding, with blockage of both receptors producing an increased effect (Fig. 2e and 2f).

HMW-HA Binding to hSiglec9 Induces SHP-1 Recruitment and Blunts Neutrophil Extracellular Trap (NET) Production, Oxidative Burst and Apoptosis

When an inhibitory CD33rSiglec engages sialoglycan ligands via its V-set domain, a signal is transduced to the cytoplasmic ITIM (immunoreceptor tyrosine-based inhibitory motif) that interacts with and activates inhibitory phosphatase *Src* homology domain 2-containing tyrosine phosphatase-1 (SHP-1) [3]. We found that HMW-HA treatment of human neutrophils increased SHP-1 association with hSiglec-9 at baseline and following stimulation with phorbol 12-myristate 13-acetate (PMA) (Fig. 3a). Upon stimulation, neutrophils generate ROS and elaborate DNA-based extracellular traps (NETs) [18]. Treatment with HMW-HA inhibited ROS production by PMA-stimulated human neutrophils, and this inhibition was counteracted by treatment with α-Sig-9(HA) but not α-Sig-9(Sia) or anti-CD44 (Fig. 3b). NETs are the byproduct of a specialized cell death process in which decondensed chromatin is released into the extracellular space, forming fibrous structures decorated with antimicrobial histones, peptides and proteases [18, 43]. Pretreatment with HMW-HA attenuated NET production upon PMA stimulation, as assessed by immunostaining of extracellular DNA/myeloperoxidase and DNA quantification, NET formation is indicated by white arrows (Fig. 3c and 3d). NET production was restored in the presence of HMW-HA by treatment with α-Sig-9(HA) but not α-Sig-9(Sia) (Fig. 3c and 3d). In contrast with its lack of effect on ROS generation, anti-CD44 also restored NET production in the presence of HMW-HA (Fig. 3c and 3d).

Neutrophils have the shortest lifespan among circulating leukocytes [44], and prolongation of neutrophil lifespan is important for effective host defense at sites of infection or tissue injury. Conversely, apoptosis and clearance of activated neutrophils is a critical control point for terminating the inflammatory response [45]. Purified human neutrophils exposed to HMW-HA exhibited a significant increase in viability after 24 h in standard RPMI media compared to control neutrophils (11.3% vs. 2.7%) (Fig. 4a); addition of α-Sig-9(HA) inhibited the protective effect of HMW-HA (Fig. 4a). When neutrophil apoptosis was assessed by TUNEL assay at 18 h in the presence or absence of the apoptosis-inducing agent staurosporine, HMW-HA was found to inhibit apoptosis in a manner that was reversible by

α-Sig-9(HA) but not by an isotype control antibody (Fig. 4b). Collectively, these data indicate that HMW-HA interaction with hSiglec-9 serves to blunt neutrophil ROS generation, NET formation and apoptosis.

Group A *Streptococcus* (GAS) Engages hSiglec-9 Via its Surface HMW-HA Capsule

CD33rSiglec function is exploited by the bacterial pathogen GBS, which expresses a preferred terminal α2–3-linked Sia (Neu5Ac) ligand in its surface polysaccharide capsule, a "molecular mimicry" that allows hSiglec-9 binding and down-regulates neutrophil responses, promoting bacterial survival [21, 22]. An even more prevalent human pathogen, GAS, causes both localized and life-threatening invasive infections, and expresses a surface capsule composed of HMW-HA, shielding it from host immune detection [46]. GAS mutants lacking HA capsule are sensitive to phagocytic killing and attenuated in animal infection models [39]. GAS strains isolated from invasive human infections or upon animal passage are frequently hyper-encapsulated due to mutations in the *covRS* (*csrRS*) system regulating HA biosynthesis [47, 48]. We found that hSiglec-9–Fc bound to GAS strain 5448 (WT), a clinical isolate representative of the globally disseminated, hyper-virulent M1T1 clone [38] (Fig. 5a). This interaction was HA-dependent, as hSiglec-9–Fc did not bind an isogenic HA-deficient mutant (Δ*hasA*) GAS (Fig. 5a), and the interaction was blocked by α-Sig-9(HA) but not α-Sig-9(Sia) (Fig. 5b). On the other hand, the binding of the sialylated bacterial pathogen group B *Streptococcus* (GBS) to hSiglec-9–Fc was blocked by using α-Sig-9(Sia), but not α-Sig-9(HA) (Fig. 5b). Conversely, 1.6-fold increase in hSiglec-9–Fc binding was observed using an animal-passaged (AP) hyper-encapsulated derivative of the WT GAS strain known to harbor a *covS* mutation (Fig. 5a). GAS binding by hSiglec-9–Fc was much more prominent that binding by hSiglec−5, −6, −7 and −11, and similar in magnitude to GAS binding by CD44–Fc (Fig. 5c). Competition with soluble HMW-HA blocked hSiglec-9–Fc recognition of WT GAS in a dose-dependent manner, whereas identical amounts of the negatively-charged sulfated GAGs heparin and heparan sulfate did not interfere with binding (Fig. 5d). These data indicate that GAS functionally interacts with hSiglec-9 via its HMW-HA capsule.

HMW-HA Mimicry by the Bacterial Pathogen Group A *Streptococcus* (GAS) Subverts Neutrophil Oxidative Burst, NETs and Bactericidal Activity

A principal role of neutrophils is to limit pathogen dissemination. We hypothesized that HA molecular mimicry by GAS could blunt neutrophil activation through engagement of the inhibitory hSiglec-9. When neutrophils were infected with WT and capsule-deficient Δ*hasA* GAS, production of ROS was significantly lower in response to the HA-expressing WT strain (Fig. 6a and 6b). The impaired neutrophil ROS response to WT GAS was partially restored by treatment with α-Sig-9(HA) but not α-Sig-9(Sia) (Fig. 6a). These antibodies had no influence on neutrophil ROS production at baseline (Fig. 6a) or in response to the acapsular Δ*hasA* strain (Fig. 6b). Tested in parallel in this assay, anti-CD44 treatment did not influence neutrophil ROS production (Fig. 6a). Since the M1T1 clone of GAS elaborates a potent DNase that rapidly degrades NETs [38, 40], we used an isogenic DNase knockout mutant (Δ*sda1*) treated or not treated with hyaluronidase (HA-ase) to determine the effect of GAS HA capsule on NET production. Significantly fewer NETs were produced in response to the capsule-expressing GAS Δ *sda1* vs. the hyaluronidase-treated GAS Δ *sda1*, an effect

that was once again counteracted by treatment with α-Sig-9(HA) but not α-Sig-9(Sia) (Fig. 6c and 6d). Antibody treatments did not restore NET production in response to HA-ase-treated GAS Δ *sda1*, suggesting that either DNase or HA are sufficient to inactivate NET defenses (Fig. 6c and 6d). Indeed, when pretreated with α-Sig-9(HA) but not α-Sig-9(Sia), human neutrophils showed significantly enhanced killing of WT HA-expressing GAS (Fig. 6e). Tested in parallel in these assays, anti-CD44 treatment also increased NET production and bacterial killing in response to infection with WT GAS (Fig. 6c, 6d and 6e). These results indicate that GAS can utilize HA mimicry to blunt neutrophil activation and promote its own survival through engagement of hSiglec-9 and CD44 on the neutrophil surface.

Murine Siglec-E Binds HMW-HA and is Exploited by Pathogen Group A *Streptococcus* Molecular Mimicry for Innate Immune Evasion

The mouse functional paralogue of hSiglec-9, mSiglecE, was found to bind HMW-HA, albeit at a reduced level compared to the human inhibitory receptor (Fig. 7a). The recent availability of mSiglec-E knockout mice [49] allowed us to further examine the significance of this receptor in GAS HMW-HA capsule-mediated resistance to neutrophil killing and innate immune clearance. Compared to WT controls, mSiglec-E-deficient neutrophils showed enhanced killing of WT GAS (Fig. 7b), and whole blood of mSiglec-E knockout mice better restricted the growth of the WT bacterium (Fig. 7b); the nonvirulent acapsular Δ*hasA* GAS did not proliferate in the blood of either mouse strain (Fig. 7c). When mice were challenged systemically with WT GAS by intraperitoneal injection and sacrificed 5 h post-infection, significantly reduced bacterial counts (approximately 1 log-fold lower) were recovered from the peritoneal fluid, liver and spleen of mSiglecE KO compared to WT animals (Fig. 7d). These findings suggest that the inhibitory neutrophil receptor mSiglecE can be exploited by the HMW-HA expressing GAS to promote its own survival, an example of molecular mimicry that recapitulates the Sia-dependent mSiglecE engagement recently shown to promotes GBS virulence in the mouse model [22].

Discussion

We have identified hSiglec-9, prominently expressed on neutrophils, as the first example of a Siglec that recognizes a glycan other than Sia. The HA binding site is located in the Ig-like V-set domain and is distinct from the Sia-binding site, and only native HMW-HA preparations (>1,000 kDa) efficiently engage hSiglec-9. CD44 is the main receptor responsible for HA recognition [33] and diverse HA-binding proteins have been identified, including brevican [50], neurocan [51], versican [52], aggrecan [53], lymphatic vessel endothelial hyaluronan receptor-1 (LYVE1) [54], TNF-stimulated gene-6 (TSG-6) [55], hyaluronan receptor for endocytosis (HARE) [56]. In addition, serum-derived hyaluronan-associated protein (SHAP) is a HA modifying protein [57], and now in our study, a prominent binding of HMW-HA by CD33-related hSiglec-9 is revealed. A motif responsible for binding to HA was present in all the earlier proteins, except for aggrecan and SHAP, designated the LINK module [58] and the B(X_7) B motif; when we mutated the essential residues in the LINK module (data not shown) or eliminated the domain that contained the LINK-like module (Fig. 1c), we did not abrogate the binding of HMW-HA to hSiglec-9–Fc

(Fig. 1c). These data suggest that the recognition of HA by hSiglec-9 is mediated by a novel HA-binding domain.

A number of studies have focused on the regulation of macrophage activation by HA, where it has been observed that native HMW-HA (>1×10^6 Da) preparations dampened inflammation by engaging CD44 [30]. However LMW-HA (< 5×10^5 Da) fragments, arising from degradation by excessive reactive oxygen species that accumulate during tissue injury and inflammation, can exacerbate the inflammatory response by interacting with Toll-like receptors [3, 24, 34]. In asthmatic patients, fibroblasts produced elevated concentrations of LMW-HA and alveolar macrophages down-regulated the expression of CD44 that impaired HA clearance from the lung, contributing to an enhanced inflammatory response [59]. Our data showed that only native HMW-HA efficiently bound Siglec-9 and LMW-HA (2–3 × 10^5 Da) could not outcompete the Siglec-9 recognition (Fig. 2a).

Limited information is known about the function of HA in neutrophil biology. HA is rare in the blood circulation because it is rapidly cleared by specific hepatic receptors [60]. One study found that neutrophils adhere within liver sinusoids via CD44, where HA is abundant, and that blocking of CD44-HA interaction diminished liver pathophysiology and damage in response to LPS challenge [61]. Cross-linking of CD44 using an anti-CD44 antibody induced IL-6 secretion in neutrophils, in a manner that was further enhanced by interferon-γ [62]. However, another study recently suggested that the adhesion of neutrophils to endothelium was HA-dependent but CD44-independent [63]. In our case, HMW-HA by engaging hSiglec-9 on neutrophils promoted recruitment of SHP-1 and limited oxidative burst, NET production and apoptosis. Recently, extraordinarily HMW-HA (up to 6 megadaltons) unique to the naked mole rat was shown to block malignant transformation via engagement of a CD44/Merlin/INK4A signaling pathway, perhaps contributing to the notably long lifespan of the species [64]. Our data suggest another possible contributory mechanism for this finding -- the dampening of production by innate immune cells of free radicals that are thought to promote aging [65].

Neutrophils are the most abundant leukocytes in the blood, and are recruited to the site of insult in response to bacterial infection within minutes. However, even in the absence of an inciting pathogen, i.e. during trauma, ischemia, perfusion, injury, toxin exposure and certain auto-inflammatory disorders, the release of DAMPs into the extracellular space can induce chemokine release and upregulation of endothelial adhesion molecules that promote neutrophil recruitment. Under these circumstances, selective mechanisms able to counter-regulate the neutrophil driven inflammatory process must exist. CD33-related Siglecs have been recognized as negative regulators of the innate immune response and leukocyte reactivity, but heretofore only through their recognition of sialic acids (Sia) [6, 7]. Like Sia, HMW-HA appears to function as a SAMP capable of modulating the neutrophil activation state (Fig. 8). Strikingly, the dual homeostatic function of hSiglec-9 has been independently exploited through parallel molecular mimicry phenotypes by two important human bacterial pathogens, GBS (Sia) and GAS (HMW-HA), an apparent example of convergent evolution to dampen neutrophil activation and increase resistance to neutrophil killing (Fig. 8). Understanding the molecular basis of specific glycan recognition by inhibitory Siglec

receptors may provide opportunities for therapeutic manipulation of neutrophil function in inflammatory and infectious disease conditions.

Acknowledgements

Major research funding was provided by the NIH/NHLBI Programs of Excellence in Glycosciences Grant P01-HL107150 (AV and VN) and by NIH/NIAID grant R01-AI077780 (VN), a UC MEXUS-CONACYT Postdoctoral Research Fellowship (IS), the UCSD/SDSU IRACDA Postdoctoral Fellowship Program (AL), and a Wenner-Gren Foundations Fellowship, Sweden (KMR).

REFERENCES

1. Crocker PR, Paulson JC, Varki A. Siglecs and their roles in the immune system. Nat Rev Immunol. 2007; 7:255–266. [PubMed: 17380156]
2. Angata T, Hingorani R, Varki NM, Varki A. Cloning and characterization of a novel mouse Siglec, mSiglec-F: differential evolution of the mouse and human (CD33) Siglec-3-related gene clusters. J Biol Chem. 2001; 276:45128–45136. [PubMed: 11579105]
3. Taylor VC, Buckley CD, Douglas M, Cody AJ, Simmons DL, Freeman SD. The myeloid-specific sialic acid-binding receptor, CD33, associates with the protein-tyrosine phosphatases, SHP-1 and SHP-2. J Biol Chem. 1999; 274:11505–11512. [PubMed: 10206955]
4. Cao H, Crocker PR. Evolution of CD33-related siglecs: regulating host immune functions and escaping pathogen exploitation? Immunology. 2011; 132:18–26. [PubMed: 21070233]
5. Varki A. Since there are PAMPs and DAMPs, there must be SAMPs? Glycan “self-associated molecular patterns” dampen innate immunity, but pathogens can mimic them. Glycobiology. 2011; 21:1121–1124. [PubMed: 21932452]
6. Crocker PR, McMillan SJ, Richards HE. CD33-related siglecs as potential modulators of inflammatory responses. Ann N Y Acad Sci. 2012; 1253:102–111. [PubMed: 22352893]
7. Pillai S, Netravali IA, Cariappa A, Mattoo H. Siglecs and immune regulation. Annu Rev Immunol. 2012; 30:357–392. [PubMed: 22224769]
8. Chen GY, Tang J, Zheng P, Liu Y. CD24 and Siglec-10 selectively repress tissue damage-induced immune responses. Science. 2009; 323:1722–1725. [PubMed: 19264983]
9. Scaffidi P, Misteli T, Bianchi ME. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature. 2002; 418:191–195. [PubMed: 12110890]
10. Davalos D, Grutzendler J, Yang G, Kim JV, Zuo Y, Jung S, Littman DR, Dustin ML, Gan WB. ATP mediates rapid microglial response to local brain injury in vivo. Nature Neurosci. 2005; 8:752–758. [PubMed: 15895084]
11. Quintana FJ, Cohen IR. Heat shock proteins as endogenous adjuvants in sterile and septic inflammation. J Immunol. 2005; 175:2777–2782. [PubMed: 16116161]
12. Zhang Q, Raoof M, Chen Y, Sumi Y, Sursal T, Junger W, Brohi K, Itagaki K, Hauser CJ. Circulating mitochondrial DAMPs cause inflammatory responses to injury. Nature. 2010; 464:104–107. [PubMed: 20203610]
13. Kumar S, Ingle H, Prasad DV, Kumar H. Recognition of bacterial infection by innate immune sensors. Crit Rev Microbiol. 2013; 39:229–246. [PubMed: 22866947]
14. Zhang M, Angata T, Cho JY, Miller M, Broide DH, Varki A. Defining the in vivo function of Siglec-F, a CD33-related Siglec expressed on mouse eosinophils. Blood. 2007; 109:4280–4287. [PubMed: 17272508]
15. Gao PS, Shimizu K, Grant AV, Rafaels N, Zhou LF, Hudson SA, Konno S, Zimmermann N, Araujo MI, Ponte EV, et al. Polymorphisms in the sialic acid-binding immunoglobulin-like lectin-8 (Siglec-8) gene are associated with susceptibility to asthma. Eur J Hum Genet. 2010; 18:713–719. [PubMed: 20087405]
16. Cheong KA, Chang YS, Roh JY, Kim BJ, Kim MN, Park YM, Park HJ, Kim ND, Lee CH, Lee AY. A novel function of Siglec-9 A391C polymorphism on T cell receptor signaling. Int Arch Allergy Immunol. 2011; 154:111–118. [PubMed: 20733319]

17. Claude J, Linnartz-Gerlach B, Kudin AP, Kunz WS, Neumann H. Microglial CD33-related Siglec-E inhibits neurotoxicity by preventing the phagocytosis-associated oxidative burst. J Neurosci. 2013; 33:18270–18276. [PubMed: 24227736]
18. Amulic B, Cazalet C, Hayes GL, Metzler KD, Zychlinsky A. Neutrophil function: from mechanisms to disease. Annu Rev Immunol. 2012; 30:459–489. [PubMed: 22224774]
19. Angata T, Varki A. Cloning, characterization, and phylogenetic analysis of siglec-9, a new member of the CD33-related group of siglecs. Evidence for co-evolution with sialic acid synthesis pathways. J Biol Chem. 2000; 275:22127–22135. [PubMed: 10801860]
20. Zhang JQ, Nicoll G, Jones C, Crocker PR. Siglec-9, a novel sialic acid binding member of the immunoglobulin superfamily expressed broadly on human blood leukocytes. J Biol Chem. 2000; 275:22121–22126. [PubMed: 10801862]
21. Carlin AF, Uchiyama S, Chang YC, Lewis AL, Nizet V, Varki A. Molecular mimicry of host sialylated glycans allows a bacterial pathogen to engage neutrophil Siglec-9 and dampen the innate immune response. Blood. 2009; 113:3333–3336. [PubMed: 19196661]
22. Chang YC, Olson J, Beasley FC, Tung C, Zhang J, Crocker PR, Varki A, Nizet V. Group B Streptococcus engages an inhibitory Siglec through sialic acid mimicry to blunt innate immune and inflammatory responses in vivo. PLoS Pathog. 2014; 10:e1003846. [PubMed: 24391502]
23. Hascall, V.; Esko, JD. Hyaluronan. In: Varki, A.; Cummings, RD.; Esko, JD.; Freeze, HH.; Stanley, P.; Bertozzi, CR.; Hart, GW.; Etzler, ME., editors. Essentials of Glycobiology. NY: Cold Spring Harbor; 2009.
24. Jiang D, Liang J, Noble PW. Hyaluronan as an immune regulator in human diseases. Physiol Rev. 2011; 91:221–264. [PubMed: 21248167]
25. Meyer K, Smyth EM, Dawson MH. The nature of the muco-polysaccharide of synovial fluid. Science. 1938; 88:129. [PubMed: 17773757]
26. Torii S, Bashey R. High content of hyaluronic acid in normal human heart valves. Nature. 1966; 209:506–507. [PubMed: 5919581]
27. Armstrong SE, Bell DR. Relationship between lymph and tissue hyaluronan in skin and skeletal muscle. Am J Physiol Heart Circ Physiol. 2002; 283:H2485–H2494. [PubMed: 12388305]
28. Juhlin L. Hyaluronan in skin. J Intern Med. 1997; 242:61–66. [PubMed: 9260568]
29. Toole BP. Hyaluronan: from extracellular glue to pericellular cue. Nat Rev Cancer. 2004; 4:528–539. [PubMed: 15229478]
30. McKee CM, Penno MB, Cowman M, Burdick MD, Strieter RM, Bao C, Noble PW. Hyaluronan (HA) fragments induce chemokine gene expression in alveolar macrophages. The role of HA size and CD44. J Clin Invest. 1996; 98:2403–2413. [PubMed: 8941660]
31. Horton MR, McKee CM, Bao C, Liao F, Farber JM, Hodge-DuFour J, Pure E, Oliver BL, Wright TM, Noble PW. Hyaluronan fragments synergize with interferon-gamma to induce the C-X-C chemokines mig and interferon-inducible protein-10 in mouse macrophages. J Biol Chem. 1998; 273:35088–35094. [PubMed: 9857043]
32. Cantor JO, Nadkarni PP. Hyaluronan: the Jekyll and Hyde molecule. Inflamm Allergy Drug Targ. 2006; 5:257–260.
33. Aruffo A, Stamenkovic I, Melnick M, Underhill CB, Seed B. CD44 is the principal cell surface receptor for hyaluronate. Cell. 1990; 61:1303–1313. [PubMed: 1694723]
34. Muto J, Yamasaki K, Taylor KR, Gallo RL. Engagement of CD44 by hyaluronan suppresses TLR4 signaling and the septic response to LPS. Mol Immunol. 2009; 47:449–456. [PubMed: 19781786]
35. Teder P, Vandivier RW, Jiang D, Liang J, Cohn L, Pure E, Henson PM, Noble PW. Resolution of lung inflammation by CD44. Science. 2002; 296:155–158. [PubMed: 11935029]
36. Carlin AF, Lewis AL, Varki A, Nizet V. Group B streptococcal capsular sialic acids interact with siglecs (immunoglobulin-like lectins) on human leukocytes. J Bacteriol. 2007; 189:1231–1237. [PubMed: 16997964]
37. Chatellier S, Ihendyane N, Kansal RG, Khambaty F, Basma H, Norrby-Teglund A, Low DE, McGeer A, Kotb M. Genetic relatedness and superantigen expression in group A streptococcus serotype M1 isolates from patients with severe and nonsevere invasive diseases. Infect Immun. 2000; 68:3523–3534. [PubMed: 10816507]

38. Walker MJ, Hollands A, Sanderson-Smith ML, Cole JN, Kirk JK, Henningham A, McArthur JD, Dinkla K, Aziz RK, Kansal RG, et al. DNase Sda1 provides selection pressure for a switch to invasive group A streptococcal infection. Nat Med. 2007; 13:981–985. [PubMed: 17632528]
39. Cole JN, Pence MA, von Kockritz-Blickwede M, Hollands A, Gallo RL, Walker MJ, Nizet V. M protein and hyaluronic acid capsule are essential for in vivo selection of covRS mutations characteristic of invasive serotype M1T1 group A *Streptococcus* . MBio. 2010; 1:e00191–e00110. [PubMed: 20827373]
40. Buchanan JT, Simpson AJ, Aziz RK, Liu GY, Kristian SA, Kotb M, Feramisco J, Nizet V. DNase expression allows the pathogen group A *Streptococcus* to escape killing in neutrophil extracellular traps. Curr Biol. 2006; 16:396–400. [PubMed: 16488874]
41. Wessels, MR.; Benedi, VJ.; Kasper, DL.; Heggen, LM.; Rubens, CE. Type III capsule and virulence of group B streptococci. In: Dunny, GM.; Cleary, PP.; McKay, LL., editors. Genetics and Molecular Biology of Streptococci, Lactococci, and Enterococci. Washington, D. C: ASM Press; 1991. p. 219-223.
42. Butler LM, Rainger GE, Nash GB. A role for the endothelial glycosaminoglycan hyaluronan in neutrophil recruitment by endothelial cells cultured for prolonged periods. Exp Cell Res. 2009; 315:3433–3441. [PubMed: 19716819]
43. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, Weinrauch Y, Zychlinsky A. Neutrophil extracellular traps kill bacteria. Science. 2004; 303:1532–1535. [PubMed: 15001782]
44. Geering B, Simon HU. Peculiarities of cell death mechanisms in neutrophils. Cell Death Differ. 2011; 18:1457–1469. [PubMed: 21637292]
45. Milot E, Filep JG. Regulation of neutrophil survival/apoptosis by Mcl-1. Sci World J. 2011; 11:1948–1962.
46. Wessels MR, Moses AE, Goldberg JB, DiCesare TJ. Hyaluronic acid capsule is a virulence factor for mucoid group A streptococci. Proc Natl Acad Sci USA. 1991; 88:8317–8321. [PubMed: 1656437]
47. Sumby P, Whitney AR, Graviss EA, DeLeo FR, Musser JM. Genome-wide analysis of group a streptococci reveals a mutation that modulates global phenotype and disease specificity. PLoS Pathog. 2006; 2:e5. [PubMed: 16446783]
48. Cole JN, Barnett TC, Nizet V, Walker MJ. Molecular insight into invasive group A streptococcal disease. Nature reviews Microbiology. 2011; 9:724–736. [PubMed: 21921933]
49. McMillan SJ, Sharma RS, McKenzie EJ, Richards HE, Zhang J, Prescott A, Crocker PR. Siglec-E is a negative regulator of acute pulmonary neutrophil inflammation and suppresses CD11b beta2-integrin-dependent signaling. Blood. 2013; 121:2084–2094. [PubMed: 23315163]
50. Jaworski DM, Kelly GM, Piepmeier JM, Hockfield S. BEHAB (brain enriched hyaluronan binding) is expressed in surgical samples of glioma and in intracranial grafts of invasive glioma cell lines. Cancer research. 1996; 56:2293–2298. [PubMed: 8625302]
51. Deepa SS, Carulli D, Galtrey C, Rhodes K, Fukuda J, Mikami T, Sugahara K, Fawcett JW. Composition of perineuronal net extracellular matrix in rat brain: a different disaccharide composition for the net-associated proteoglycans. J Biol Chem. 2006; 281:17789–17800. [PubMed: 16644727]
52. Matsumoto K, Shionyu M, Go M, Shimizu K, Shinomura T, Kimata K, Watanabe H. Distinct interaction of versican/PG-M with hyaluronan and link protein. J Biol Chem. 2003; 278:41205–41212. [PubMed: 12888576]
53. Seyfried NT, McVey GF, Almond A, Mahoney DJ, Dudhia J, Day AJ. Expression and purification of functionally active hyaluronan-binding domains from human cartilage link protein, aggrecan and versican: formation of ternary complexes with defined hyaluronan oligosaccharides. J Biol Chem. 2005; 280:5435–5448. [PubMed: 15590670]
54. Banerji S, Ni J, Wang SX, Clasper S, Su J, Tammi R, Jones M, Jackson DG. LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. J Cell BIol. 1999; 144:789–801. [PubMed: 10037799]

55. Kahmann JD, O'Brien R, Werner JM, Heinegard D, Ladbury JE, Campbell ID, Day AJ. Localization and characterization of the hyaluronan-binding site on the link module from human TSG-6. Structure. 2000; 8:763–774. [PubMed: 10903951]

56. Politz O, Gratchev A, McCourt PA, Schledzewski K, Guillot P, Johansson S, Svineng G, Franke P, Kannicht C, Kzhyshkowska J, et al. Stabilin-1 and −2 constitute a novel family of fasciclin-like hyaluronan receptor homologues. Biochem J. 2002; 362:155–164. [PubMed: 11829752]

57. Huang L, Yoneda M, Kimata K. A serum-derived hyaluronan-associated protein (SHAP) is the heavy chain of the inter alpha-trypsin inhibitor. J Biol Chem. 1993; 268:26725–26730. [PubMed: 7504674]

58. Kohda D, Morton CJ, Parkar AA, Hatanaka H, Inagaki FM, Campbell ID, Day AJ. Solution structure of the link module: a hyaluronan-binding domain involved in extracellular matrix stability and cell migration. Cell. 1996; 86:767–775. [PubMed: 8797823]

59. Liang J, Jiang D, Jung Y, Xie T, Ingram J, Church T, Degan S, Leonard M, Kraft M, Noble PW. Role of hyaluronan and hyaluronan-binding proteins in human asthma. J Allergy Clin Immunol. 2011; 128:403–411. e403. [PubMed: 21570715]

60. Harris EN, Weigel JA, Weigel PH. Endocytic function, glycosaminoglycan specificity, and antibody sensitivity of the recombinant human 190-kDa hyaluronan receptor for endocytosis (HARE). J Biol Chem. 2004; 279:36201–36209. [PubMed: 15208308]

61. McDonald B, McAvoy EF, Lam F, Gill V, de la Motte C, Savani RC, Kubes P. Interaction of CD44 and hyaluronan is the dominant mechanism for neutrophil sequestration in inflamed liver sinusoids. J Exp Med. 2008; 205:915–927. [PubMed: 18362172]

62. Sconocchia G, Campagnano L, Adorno D, Iacona A, Cococcetta NY, Boffo V, Amadori S, Casciani CU. CD44 ligation on peripheral blood polymorphonuclear cells induces interleukin-6 production. Blood. 2001; 97:3621–3627. [PubMed: 11369659]

63. Alam CA, Seed MP, Freemantle C, Brown J, Perretti M, Carrier M, Divwedi A, West DC, Gustafson S, Colville-Nash PR, et al. The inhibition of neutrophil-endothelial cell adhesion by hyaluronan independent of CD44. Inflammopharmacology. 2005; 12:535–550. [PubMed: 16259720]

64. Tian X, Azpurua J, Hine C, Vaidya A, Myakishev-Rempel M, Ablaeva J, Mao Z, Nevo E, Gorbunova V, Seluanov A. High-molecular-mass hyaluronan mediates the cancer resistance of the naked mole rat. Nature. 2013; 499:346–349. [PubMed: 23783513]

65. Holmstrom KM, Finkel T. Cellular mechanisms and physiological consequences of redox-dependent signalling. Nature reviews Molecular cell biology. 2014; 15:411–421. [PubMed: 24854789]

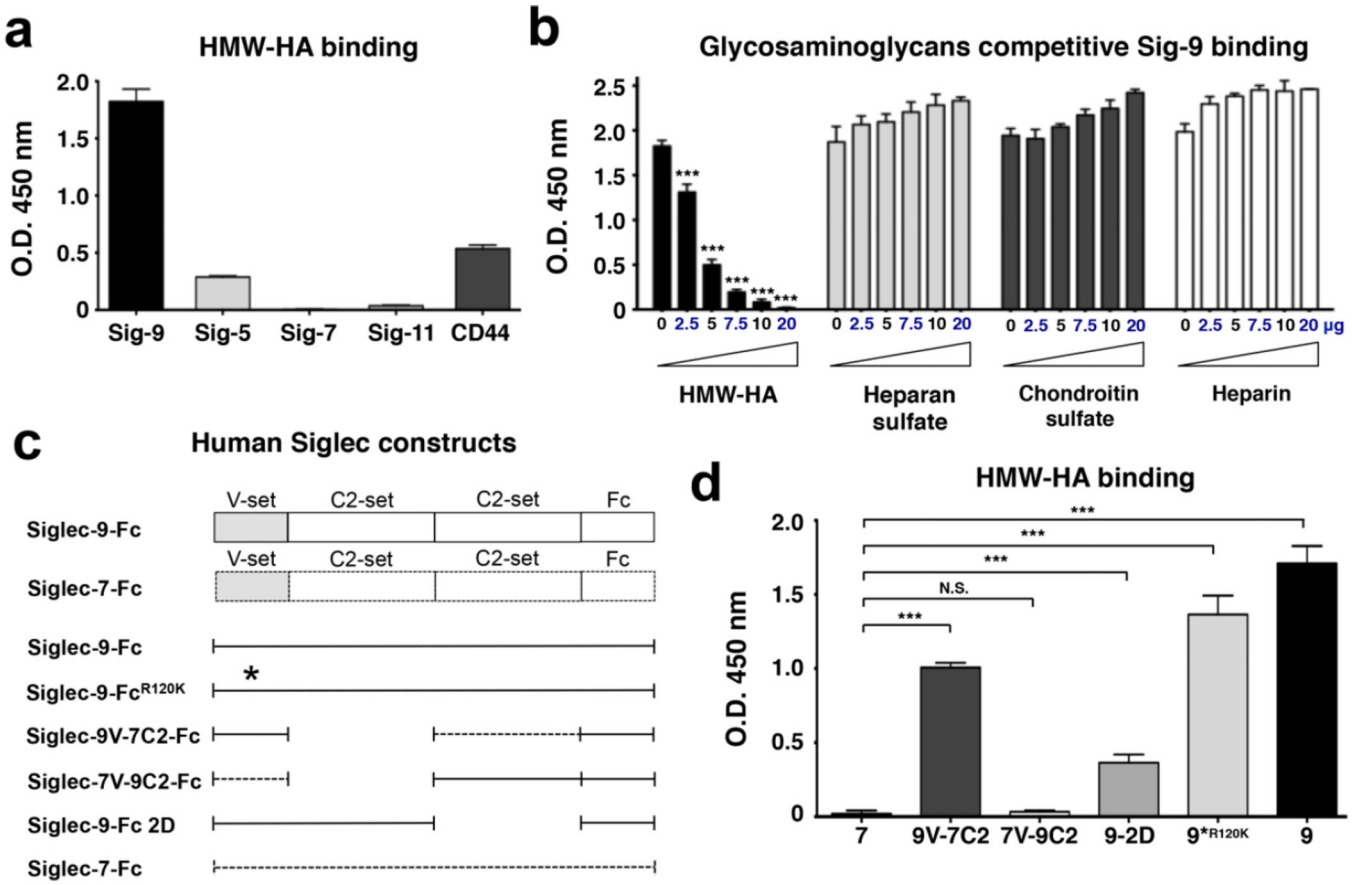

Fig 1.

Specific recognition of hyaluronan by the Ig-like-V-set domain of hSiglec-9. (**a**) Immobilized high molecular weight-hyaluronan (HMW-HA) was probed with human Siglec–Fc and CD44–Fc chimeras and binding evaluated by using an anti-human IgG-HRP. (**b**) Binding of hSiglec-9–Fc to immobilized HMW-HA was performed in the presence of increasing concentrations of HMW-HA, heparan sulfate, chondroitin sulfate or heparin. (**c, d**) To map the hSiglec-9 domain responsible for HA recognition, binding of hSiglec-9–Fc to immobilized HMW-HA was compared to binding of hSiglec-9–Fc with an Arg→Ala mutation in the V-set domain (hSiglec-9^{R120K}), a fusion protein construct of the hSiglec-9 V-set domain + the second Ig-like domain (C2-set) of hSiglec-7 + human IgG Fc tail (hSiglec-9V-7C2–Fc), a fusion protein construct encompassing V-set domain of hSiglec-7 + C2-set of hSiglec-9 + human IgG Fc tail (Siglec-7V-9C2–Fc), a fusion protein construct of the hSiglec-9 V-set domain + first C2-set domain + human IgG Fc tail (Siglec-9–Fc 2D). Results are expressed as the mean ± SD. All experiments were performed in triplicate, repeated 3 times (**a, d**) or 2 times (**b**). One-way ANOVA with Dunnett's multiple comparison test; $P < 0.001$ (***).

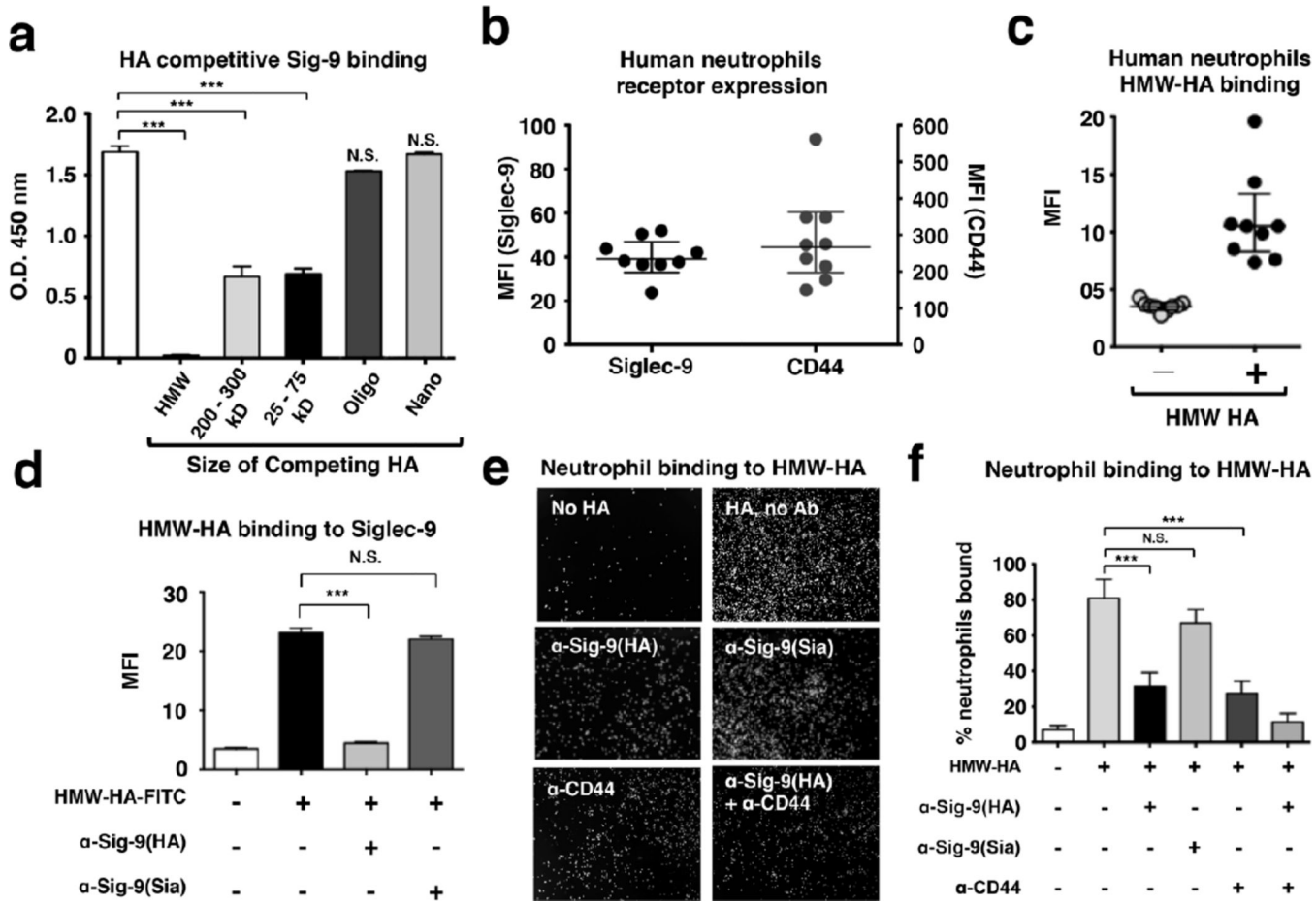

Fig 2.

High molecular weight hyaluronan (HMW-HA) is recognized by hSiglec-9 on human neutrophils. (**a**) To evaluate the molecular size of hyaluronan (HA) responsible for binding to hSiglec-9, high molecular weigh HA (HMW-HA; >1,000 kDa), low molecular weigh (200 – 300 kDa-HA and 25 – 75 kDa-HA), nano-HA (nonamers) and oligo-HA (hexamers) fragments were added to compete with binding of hSiglec-9–Fc to immobilized HMW-HA plates. Binding was evaluated using an anti-human IgG-HRP. Experiment performed in triplicate and repeated 3 times; results are expressed as mean ± SD. (**b**) Flow cytometry reveals constitutive expression of Siglec-9 and CD44 on human neutrophils from 9 different donors; geometric mean ± 95% confidence interval. (**c**) Binding of FITC-labeled HMW-HA to neutrophils of these donors was evaluated by flow cytometry; geometric mean ± 95% confidence interval. (**d**) Human neutrophils were pretreated with anti-Siglec-9 monoclonal antibodies, anti-human CDw329 (BD Pharmingen, #550906) and anti-human Siglec-9 (R&D Systems, #BAF1139) and effects on binding to FITC-labeled HMW-HA determined. CDw329 Ab blocked binding of HMW-HA, but not GBS capsule (Fig. 5b) and was designated “α-Sig-9(HA)”; in contrast, the R&D Systems Ab blocked recognition of GBS capsule (Fig. 5b), but did not interfere with binding to HMW-HA, and was thus designated “α-Sig-9(Sia)”. Data pooled from five independent experiments in triplicate; data represent the mean ± SD. (**e, f**) Calcein-labeled human neutrophils were pretreated with α-Sig-9(HA),

α-Sig-9(Sia) and α-CD44 mAbs, added to wells coated with immobilized HMW-HA to facilitate adherence, then unbound neutrophils washed away. Remaining neutrophils were lifted and enumerated. Experiment performed in triplicate and repeated 5 times; results are expressed as mean ± SD. One-way ANOVA with Dunnett's multiple comparison test; $P < 0.001$ (***).

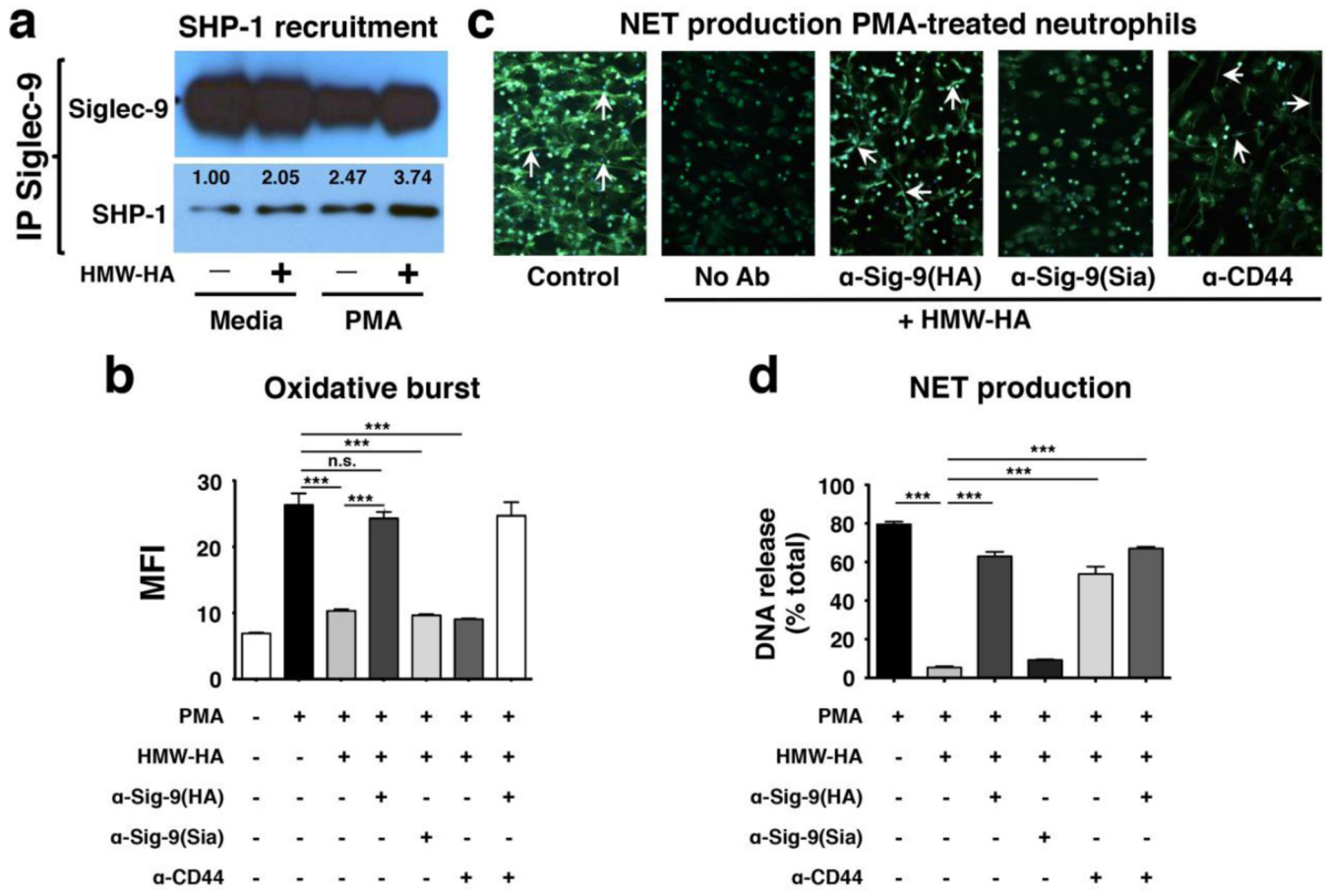

Fig 3.

HMW-HA binding to hSiglec-9 induces SHP-1 recruitment and blunts neutrophil NET production and oxidative burst. (**a**) 1.2×10^7 human neutrophils were incubated for 30 min + 10 μg/ml of high molecular weight hyaluronan (HMW-HA) + 25 nM PMA. Cell lysates were immunoprecipitated with α-Siglec9 and SHP-1 recruitment was visualized by western blot analysis; results were repeated 2 times with similar results; representative experiment with relative densitometry values is shown. (**b**) Neutrophils were pretreated with α-Sig-9(HA), α-Sig-9(Sia) or α-CD44 Abs, incubated with 10 μg/ml of HMW-HA and activated for 30 min with PMA. Reactive oxygen species (ROS) release was measured with the OxyBURST Green H_2HFF BSA probe and results expressed as mean fluorescence intensity (MFI) ± SD; experiment repeated 5 times with similar results; representative experiment is shown. (**c**) Neutrophils were pretreated α-Sig-9(HA), α-Sig-9(Sia), or α-CD44 mAbs, then incubated with 10 μg/ml of HMW-HA and activated with PMA for 3 h; production of neutrophil extracellular traps (NETs) visualized by staining for DAPI (DNA, blue) + anti-myeloperoxidase/AlexaFluor488 (green); representative fields at 20x magnification is shown; experiment performed in triplicate and repeated 5 times. (**d**) NET production was quantified by Quant-iT™ PicoGreen® assay for extracellular DNA; results are expressed as mean ± SD; experiment repeated 3 times in triplicate with similar results; representative experiment shown. One-way ANOVA with Dunnett's multiple comparison test; $P < 0.001$ (***).

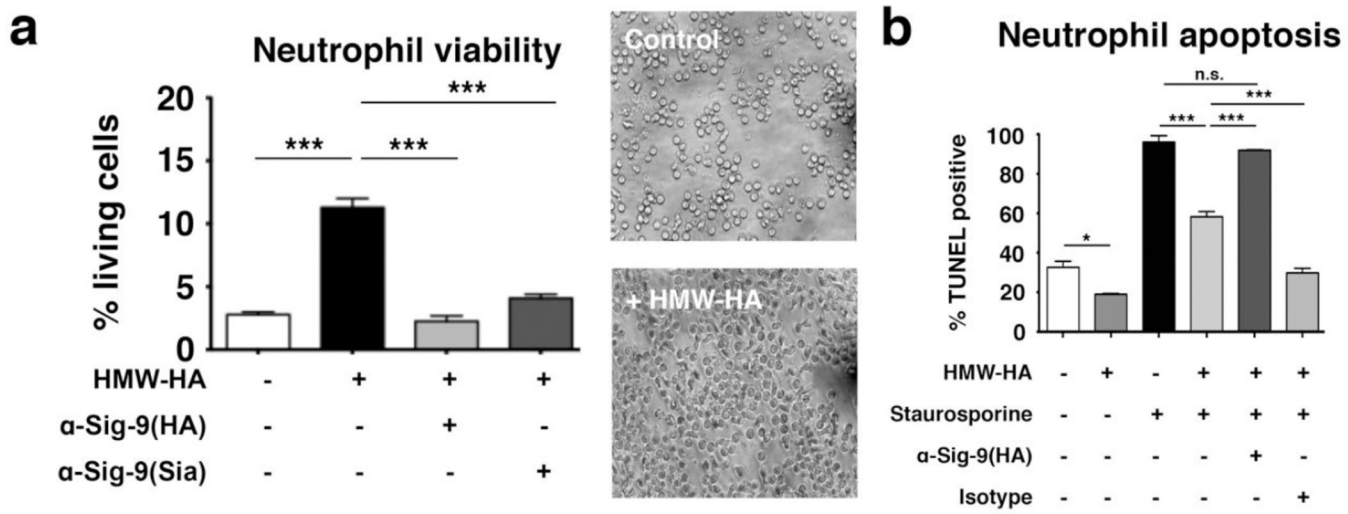

Fig 4.

HMW-HA binding to hSiglec-9 prolongs neutrophil viability and inhibits neutrophil apoptosis. (**a**) Lifespan of human neutrophils was evaluated in the presence or absence of α-Sig-9(HA) or α-Sig-9(Sia) mAbs; neutrophil viability was evaluated by trypan dye exclusion at 24 h of incubation. Results represent mean ± SD. Triplicate wells repeated 3 times with similar results; representative experiment is shown. Images show representative wells at 32x magnification. (**b**) Apoptosis of human neutrophils was evaluated upon pretreatment with α-Sig-9(HA) or isotope control IgG + 5 μg/ml of staurosporine; fragmentation of DNA was evaluated by TUNEL assay at 18 h; results expressed as mean fluorescence intensity (MFI) ± SD and repeated 2 times in triplicate with similar results; representative experiment is shown. One-Way ANOVA with Dunnett's multiple comparison test; $P < 0.001$ (***) or $P < 0.05$ (*).

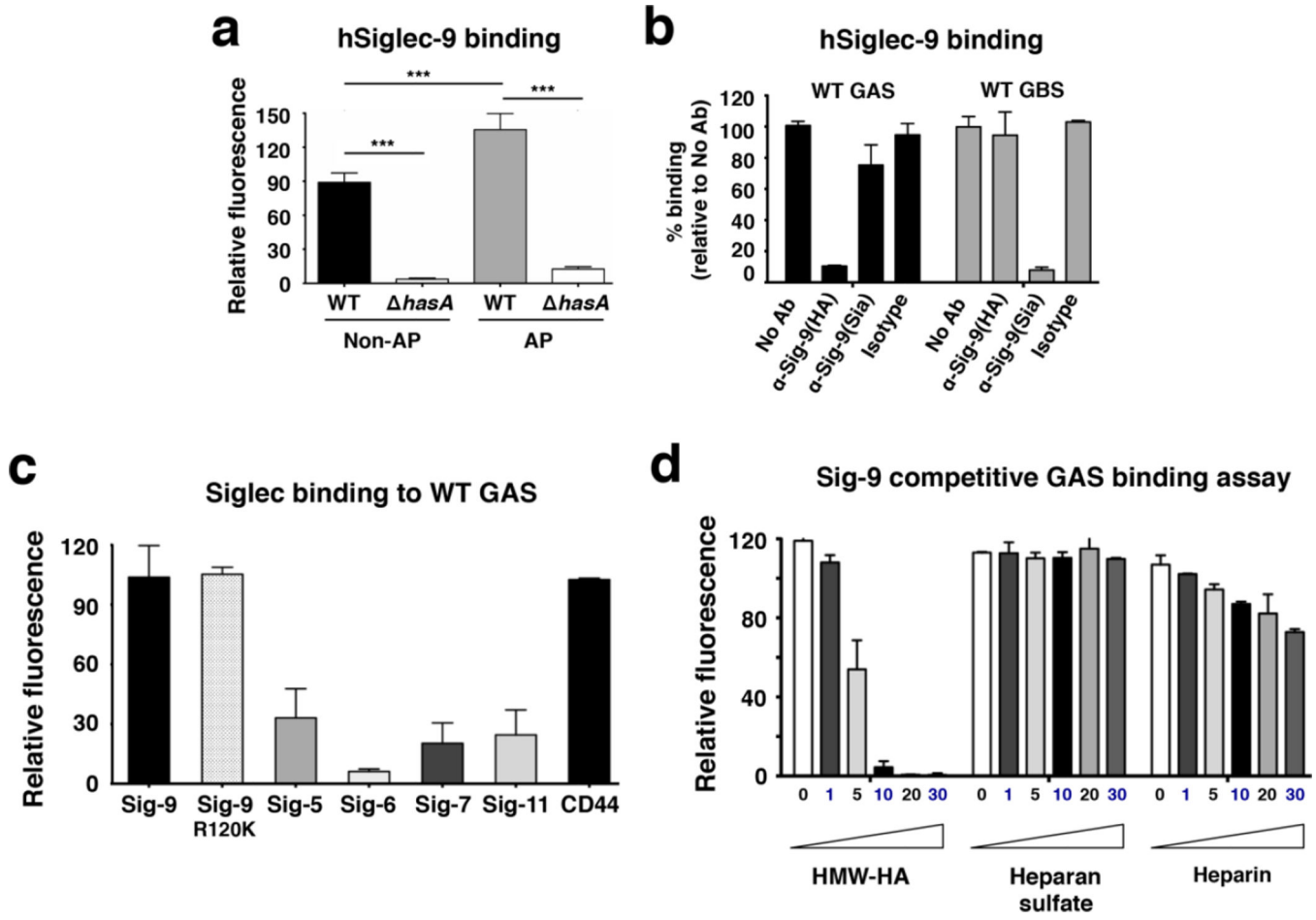

Fig 5.

Group A *Streptococcus* (GAS) engages hSiglec-9 via its surface hyaluronan capsule. (**a**) Human Siglec-9–Fc was immobilized to ELISA wells using protein A, and binding of FITC-labeled forms of WT GAS, its isogenic HA capsule-deficient mutant (Δ*hasA*), an animal-passaged hyperencapsulated (AP) derivative and its isogenic HA capsule-deficient mutant (AP Δ*hasA*) were evaluated; results are expressed as mean ± SD and repeated 5 times in triplicate with similar results; representative experiment shown. One-Way ANOVA with Dunnett's multiple comparison test; $P < 0.001$ (***) (**b**) Human Siglec-9–Fc chimera was immobilized to ELISA wells via protein A in the presence of α-Sig-9(HA), α-Sig-9(Sia) or isotype control Abs and binding of FITC-labeled WT GAS or sialic acid-expressing serotype III group B *Streptococcus* (GBS) evaluated. (**c**) Human Siglec-9/9R120K/5/6/7/11 and human CD44-Fc chimera were immobilized to ELISA wells via protein A. Binding of FITC-labeled WT GAS was evaluated. Results represent mean ± SD; triplicate wells, representative experiment depicted of 5 independent repeats with similar results, performed in triplicate. (**d**) hSiglec-9–Fc was immobilized to ELISA plates using protein A, then wells were pretreated with HMW-HA, HMW-heparan sulfate or heparin over the indicated range of concentrations. Binding of FITC-labeled WT GAS was evaluated.

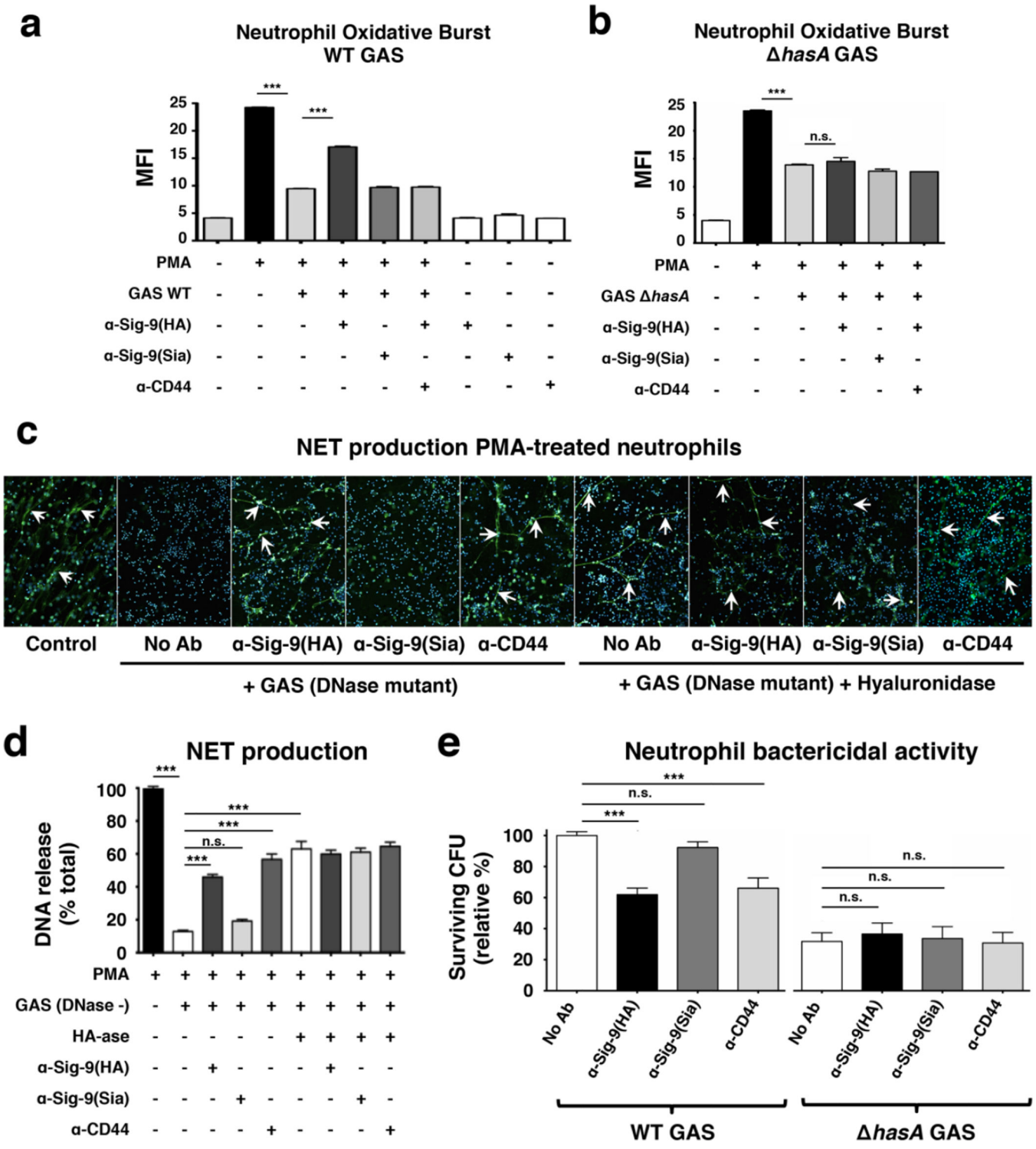

Fig 6.

Group A *Streptococcus* (GAS) binding to hSiglec-9 via its surface HMW-HA capsule blunts neutrophil oxidative burst, NET responses and bactericidal activity. (**a, b**) Neutrophils were labeled with OxyBURST Green H_2HFF BSA in the presence of α-Sig-9(HA), α-Sig-9(Sia)

or α-CD44 mAbs, infected with WT or isogenic Δ*hasA* GAS at MOI = 20 for 30 min and oxidative burst measured by FACS; results are expressed as MFI ±SD and repeated twice with similar results; representative experiment is shown. (**c**) PMA-stimulated neutrophils (5×10^5 cells) were pretreated with α-Sig-9(HA), α-Sig-9(Sia), or α-CD44 mAbs and exposed for 3 h to MOI = 10 of GAS (DNase mutant) that had been pretreated or not with hyaluronidase to remove HA capsule and NET production visualized by staining for DAPI (DNA, blue) + anti-myeloperoxidase/AlexaFluor488 (green); results are repeated 5 times in triplicate, representative fields at 32 × magnification is shown. (**d**) NET production was quantified by Quant-iT™ PicoGreen® assay for extracellular DNA; results are expressed as mean ± SD and repeated 2 times with similar results; representative experiment shown. (**e**) Neutrophils were pretreated with α-Sig-9(HA), α-Sig-9(Sia) or α-CD44 mAbs, infected with WT or isogenic Δ*hasA* GAS at multiplicity of infection (MOI) = 10 for 30 min, then cells lysed and dilutions plated on agar for enumeration of colony forming units to evaluate neutrophil killing of GAS. Data represent the mean + SD of triplicates; repeated 4 times with similar results; representative experiment shown. One-Way ANOVA with Dunnett's multiple comparison test; $P < 0.001$ (***) or $P < 0.05$ (*).

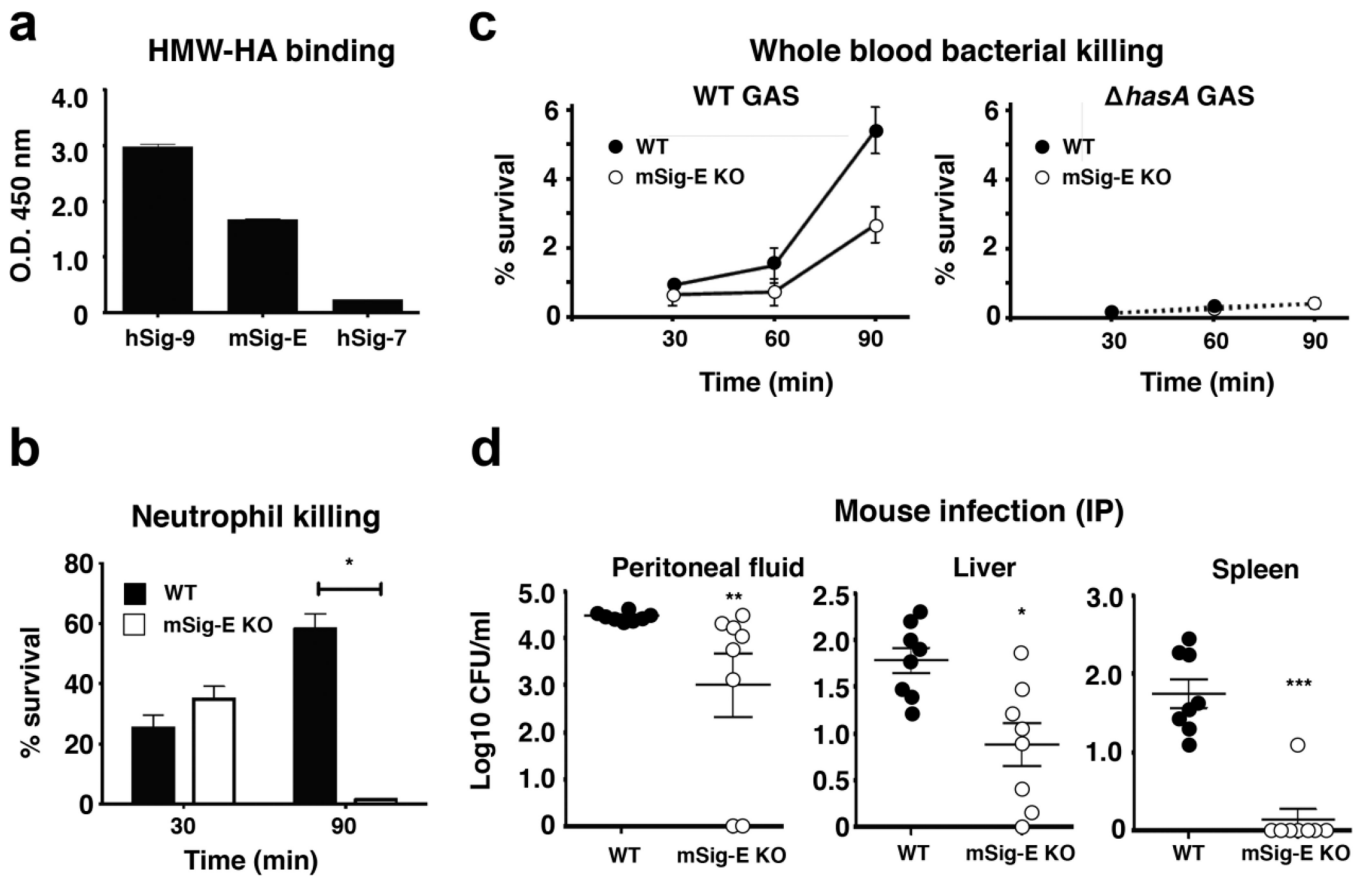

Fig 7.

Murine Siglec-E binds HMW-HA and is exploited by group A *Streptococcus* molecular mimicry for innate immune evasion. (**a**) ELISA shows mSiglec-E binds HMW-HA, at a reduced level compared to the human inhibitory receptor (n = 4 replicates). Compared to WT controls, mSiglec-E-deficient neutrophils (**b**) and whole blood (**c**) showed enhanced killing of WT GAS. Assays performed in triplicate, repeated three times for WT GAS and two times for Δ*hasA* acapsular mutant bacteria. Two-tailed t-test was used to calculate significance. (**d**) Intraperitoneal challenge of WT and mSiglec-E KO mice. 5 h post-infection, significantly reduced bacterial counts were recovered from the peritoneal fluid, liver and spleen of mSiglecE KO compared to WT animals. $n = 8$ animals per group. Statistical analysis performed by one-way ANOVA with Bonferroni post-test. $P < 0.001$ (***), $P < 0.01$ (***) or $P < 0.05$ (*)

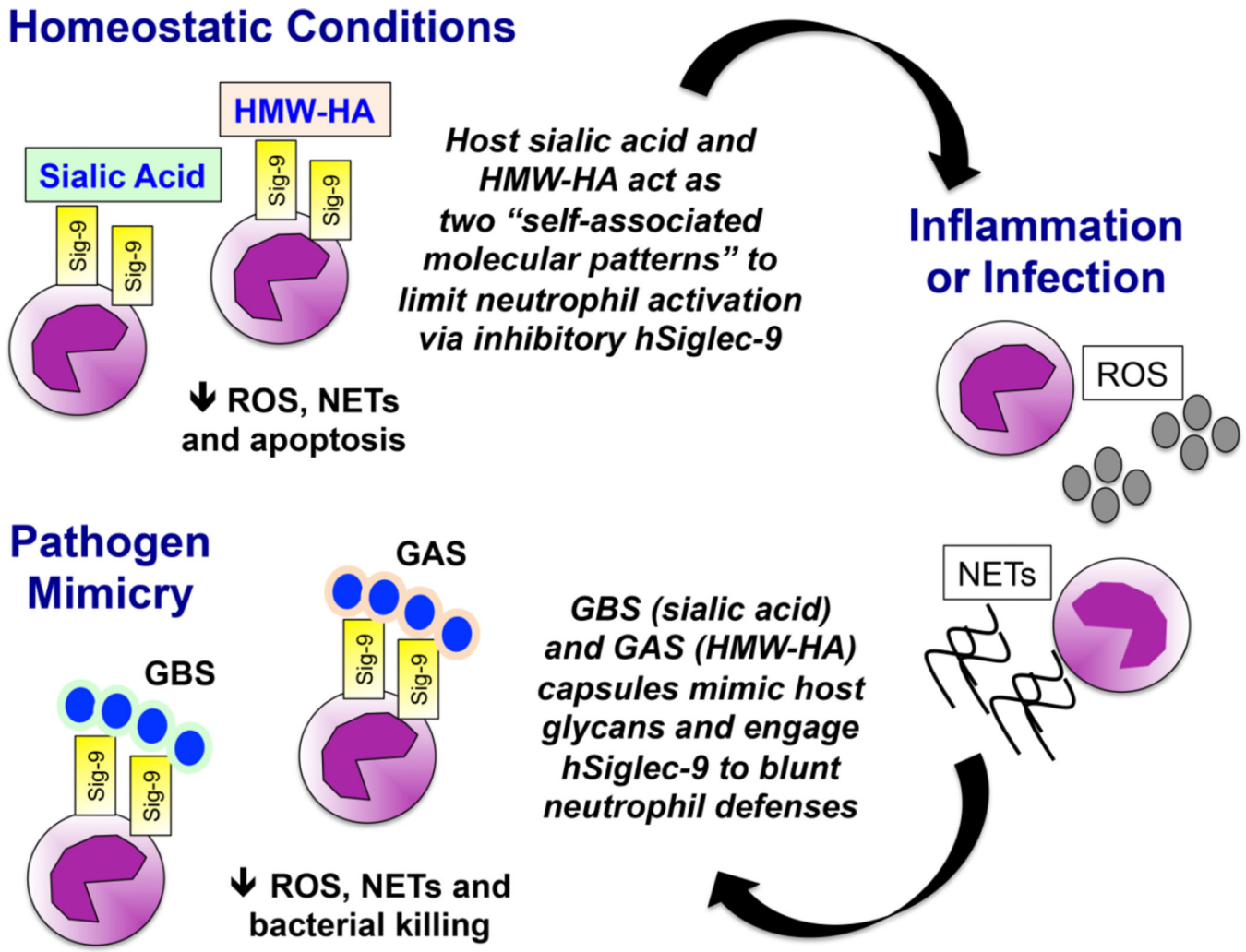

Fig 8.

Summary model. A single inhibitory receptor, human Siglec-9, detects two distinct host glycans, sialic acid and high-molecular weight hyaluronic acid (HMW-HA), as "self-associated molecular patterns" to maintain neutrophil homeostasis. Two leading human bacterial pathogens, group B *Streptococcus*, which expresses a sialic acid capsule, and group A *Streptococcus*, which expresses an HMW-HA capsule, have independently evolved molecular mimicry to exploit this immune regulatory mechanism.