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RESEARCH ARTICLE

Leukocyte Inflammatory Responses Provoked by Pneumococcal Sialidase

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ABSTRACT Cell surface expression of sialic acid has been reported to decrease during immune cell activation, but the significance and regulation of this phenomenon are still being investigated. The major human bacterial pathogen *Streptococcus pneumoniae* causes pneumonia, sepsis and meningitis, often accompanied by strong inflammatory responses. *S. pneumoniae* expresses a sialidase (NanA) that contributes to mucosal colonization, platelet clearance, and blood-brain barrier penetration. Using wild-type and isogenic NanA-deficient mutant strains, we showed that *S. pneumoniae* NanA can desialylate the surface of human THP-1 monocytes, leading to increased ERK phosphorylation, NF- κ B activation, and proinflammatory cytokine release. *S. pneumoniae* NanA expression also stimulates interleukin-8 release and extracellular trap formation from human neutrophils. A mechanistic contribution of unmasking of inhibitory Siglec-5 from *cis* sialic acid interactions to the proinflammatory effect of NanA is suggested by decreased SHP-2 recruitment to the Siglec-5 intracellular domain and RNA interference studies. Finally, NanA increased production of proinflammatory cytokines in a murine intranasal challenge model of *S. pneumoniae* pneumonia.

IMPORTANCE Sialic acids decorate the surface of all mammalian cells and play important roles in physiology, development, and evolution. Sigmals are sialic acid-binding receptors on the surface of immune cells, many of which engage in *cis* interactions with host sialoglycan ligands and dampen inflammatory responses through transduction of inhibitory signals. Recently, certain bacterial pathogens have been shown to suppress leukocyte innate immune responses by molecular mimicry of host sialic acid structures and engagement of inhibitory Sigmals. Our present work shows that the converse can be true, i.e., that a microbial sialic acid-cleaving enzyme can induce proinflammatory responses, which are in part mediated by unmasking of an inhibitory Siglec. We conclude that host leukocytes are poised to detect and respond to microbial sialidase activity with exaggerated inflammatory responses, which could be beneficial or detrimental to the host depending on the site, stage and magnitude of infection.

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Sialic acids are nine-carbon sugars prominently displayed as terminal monosaccharides on surface expressed glycoconjugates of all mammalian cells (1, 2). Sialic acids serve key roles in a diverse array of physiological and pathological processes, including organ development, immune regulation, microbial binding, malignancy, and aspects of human evolution (1, 2). An important facet of sialic acid biology is the function of immunoreceptors called sialic acid-binding immunoglobulin-like lectins, or Sigmals, which are differentially expressed across the major leukocyte lineages (3–5). A subset of these are the CD33-related Sigmals (CD33rSigmals), whose extracellular sialic binding domains are typically paired with a cytoplasmic domain containing both a membrane-proximal immunoreceptor tyrosine-based inhibitory motif (ITIM) and a membrane-distal ITIM-like motif (3, 5).

Two inhibitory CD33rSigmals expressed prominently on monocytes/macrophages and neutrophils are Siglec-5 and Siglec-9, which recruit phosphatases SHP-1 and SHP-2 to their ITIM and ITIM-like cytoplasmic domains, thereby antagonizing kinase-dependent activation cascades (6). Since the local concen-

tration of sialic acids on surfaces of leukocytes is very high, perhaps exceeding 100 mM (7), Siglec binding sites are typically “masked” by *cis* interactions with other sialoglycan ligands expressed on the same cell and can be unmasked by sialidase treatment (8). The widespread expression of host sialic acids and the prominence of cognate ITIM-bearing CD33rSigmals on innate immune cells suggest that they may function in “self-recognition” as “self-associated molecular patterns,” dampening innate immune responses to prevent autoreactivity (3, 5, 9).

A number of bacteria synthesize sialic acids or acquire them from the host environment and incorporate them into cell wall or surface components such as lipooligosaccharides (LOS), lipopolysaccharides (LPS), or capsular polysaccharides (CPS) (10). In some cases, “molecular mimicry” of host sialic acid epitopes allows the pathogen to engage inhibitory Sigmals, contributing to virulence by suppressing immune responses or altering leukocyte differentiation programs. For example, the sialylated CPS of group B streptococcus can engage Siglec-9 to suppress neutrophil activation (11–13) and the sialylated LOS of *Campylobacter jejuni*

can bind Siglec-7 to influence dendritic cell-mediated T cell responses to Th1 or Th2 polarization (14, 15).

In contrast, numerous microbial pathogens express enzymes that cleave sialic acid (sialidases; also called neuraminidases) (16, 17), such as the viruses causing influenza (18) or mumps (19), the bacterial pathogens *Streptococcus pneumoniae* (20) and *Pseudomonas aeruginosa* (21), the fungus *Aspergillus fumigatus* (22), and the protozoan parasite *Trypanosoma cruzi* (23). The overall extent of cell surface sialylation has been found to decrease in activated macrophages, neutrophils, B cells, T cells, and natural killer cells (24–28), suggesting that “unmasking” of Siglecs from *cis* sialic acid engagement occurs during immune activation (27). The processes that dynamically regulate Siglec unmasking are unknown but may in part involve endogenous mammalian sialidases (8), such as that encoded by the *neu-1* gene located within the major histocompatibility complex in humans (26, 29). A potential role of sialidases produced by microbial pathogens in Siglec unmasking has not been studied.

We hypothesized that if bacterial sialic acid mimicry could suppress host innate immune cell responses via engagement of inhibitory Siglecs, then cell surface desialylation by a bacterial sialidase could have contrasting effects. *S. pneumoniae* is the leading human bacterial pathogen causing upper respiratory tract infections, pneumonia, sepsis and meningitis, resulting in over a million deaths worldwide each year (30). To establish an experimental model for testing our hypothesis, we used isogenic strains of *S. pneumoniae* expressing or lacking their major sialidase, the surface-anchored NanA. The effect of *S. pneumoniae* sialidase expression on proinflammatory cytokine responses was examined in a human monocyte cell line and in primary neutrophils and then extended to a murine model of pneumococcal pneumonia. We document a proinflammatory effect of *S. pneumoniae* desialylation of the leukocyte cell surface associated with ERK phosphorylation and NF- κ B activation. Evidence is provided that unmasking of Siglec-5, the inhibitory CD33rSiglec, is a contributing factor to this phenomenon.

RESULTS

***S. pneumoniae* sialidase (NanA) mediates cell surface desialylation and increases monocyte cytokine responses.** NanA from *S. pneumoniae* has been shown to desialylate human airway epithelial cell glycoconjugates *in vitro* (31). We tested whether infection of human monocytic cells with wild-type (WT) *S. pneumoniae* led to general cell surface desialylation. As shown in Fig. 1A, *S. pneumoniae*-infected THP-1 cells exhibited a clear dose-dependent increase in *Erythrina cristagalli* agglutinin lectin binding, indicating removal of terminal sialic acids and exposure of underlying Gal β 1-4GlcNAc β 1 units. The isogenic sialidase-deficient *S. pneumoniae* mutant (Δ NanA) did not demonstrate such activity at the same multiplicity of infection (MOI), confirming that the increased *E. cristagalli* agglutinin binding was due to sialidase action. THP-1 cells were then challenged with WT or Δ NanA *S. pneumoniae* for different time intervals, and the release of proinflammatory cytokines into the culture supernatant was determined by enzyme-linked immunosorbent assay (ELISA). A significant increase in secretion of tumor necrosis factor alpha (TNF- α), interleukin-6 (IL-6), and IL-8 from *S. pneumoniae*-infected THP-1 monocytes compared to Δ NanA mutant-infected monocytes was observed 2 h postinfection (Fig. 1B). Similar findings were observed for TNF- α production with the U937 human

monocytic cell line (data not shown). Augmented cytokine responses were not a byproduct of differing growth or survival of WT *S. pneumoniae* versus Δ NanA *S. pneumoniae*, because THP-1 displayed no obvious bactericidal activity and no differences in THP-1 phagocytosis between the *S. pneumoniae* and Δ NanA mutant strains were found (data not shown). Complementation of the *S. pneumoniae* Δ NanA mutant with the NanA enzyme expressed on a plasmid vector partially restored the cytokine stimulation phenotype; in contrast, complementation of the Δ NanA mutant with an enzymatically inactive version of NanA (32) had no effect (Fig. 1C). These observations indicate that *S. pneumoniae* NanA is required for monocyte cell surface desialylation and that the enzymatic activity is associated with elevated proinflammatory cytokine responses. Addition of recombinant purified *Arthrobacter ureafaciens* sialidase did not itself augment THP-1 monocyte production of TNF- α ; neither did it restore the TNF- α induced by the Δ NanA mutant to the levels induced by the WT or pNanA-complemented mutant strains (Fig. 1D). Also, purified recombinant *S. pneumoniae* NanA enzyme (from QA-Bio) did not induce increased release of TNF- α from unstimulated or lipoteichoic acid (LTA)-stimulated THP-1 monocytes (Fig. 1E). Thus, the action of NanA desialylation to lower the activation threshold of THP-1 cells occurs only in the context of the live *S. pneumoniae* infection and is perhaps mediated by desialylation events triggered by surface-anchored NanA at the site of bacterium-host cell engagement.

***S. pneumoniae* NanA-stimulated monocyte supernatants increase hBMEC permeability.** During sepsis and meningitis, overactivation of inflammatory monocytes/macrophages is felt to play an important role in endothelial barrier dysfunction (33), and increased blood-brain barrier permeability and meningeal TNF- α levels are found in *S. pneumoniae*-challenged mice (34). We observed that supernatants from WT *S. pneumoniae*-infected THP-1 cells significantly increased the permeability of human brain microvascular endothelial cell (hBMEC) monolayers by a large indicator protein (horseradish peroxidase [HRP]) compared to the supernatants collected from uninfected or Δ NanA mutant-infected THP-1 cells (Fig. 1F). Neither purified supernatant from WT *S. pneumoniae* bacteria (grown in the absence of THP-1 cells) nor recombinant NanA itself was sufficient to induce hBMEC permeability (Fig. 1G). These findings suggest that NanA-mediated surface desialylation can increase monocyte release of proinflammatory cytokines, with potential downstream effects on endothelial cell permeability.

***S. pneumoniae* sialidase NanA increases neutrophil IL-8 secretion and neutrophil extracellular trap (NET) formation.** Neutrophils are critical first-line effector immune cells in the innate host response to bacterial infection. As shown in Fig. 2A, WT *S. pneumoniae* induced significantly more IL-8 secretion from human neutrophils than did the isogenic Δ NanA mutant. Neutrophil extracellular traps (NETs) are networks of extracellular fibers composed of DNA, histones, and embedded antimicrobial peptides and enzymes capable of capturing and killing bacterial pathogens (35, 36). Using a myeloperoxidase stain to aid in visualization and quantification of NETs, we found that WT *S. pneumoniae* induced more NET formation than the Δ NanA mutant (Fig. 2B and C). Thus, *S. pneumoniae* sialidase production enhances innate immune activation of neutrophils in a fashion analogous to our observations in THP-1 monocytes.

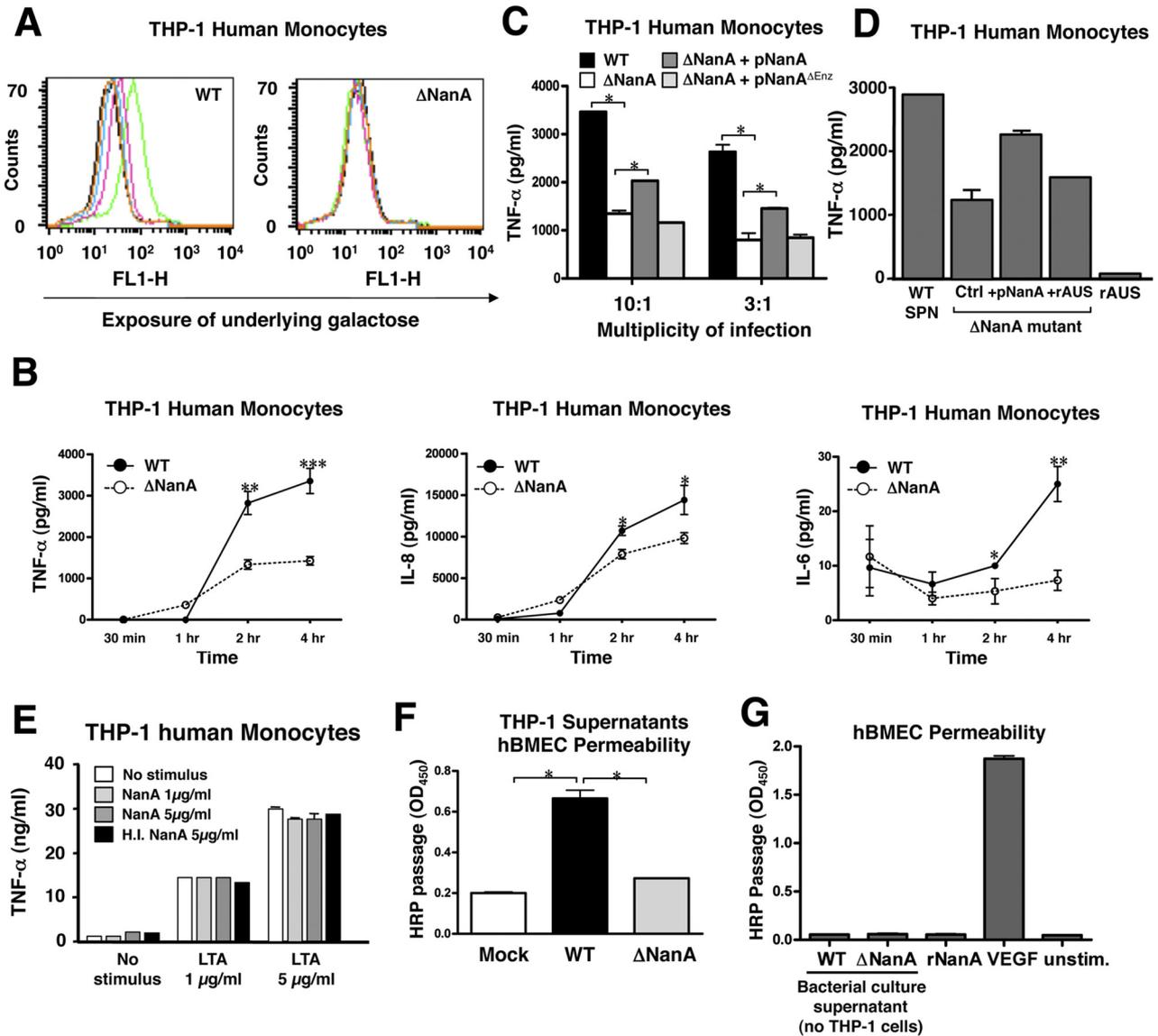


FIG 1 *S. pneumoniae* sialidase (NanA) mediates cell surface desialylation and increases monocyte cytokine responses. Surface desialylation was determined by FITC-conjugated *E. cristagalli* agglutinin staining of human THP-1 monocytes infected with WT *S. pneumoniae* or Δ NanA mutant for 3 h. Green line, MOI = 10; pink line, MOI = 3; blue line, MOI = 1. (B) Time course analysis of proinflammatory cytokine production. THP-1 cells were infected with WT *S. pneumoniae* or Δ NanA mutant at an MOI = 10 for 30 min or 1, 2, or 4 h, and the amount of IL-6, IL-8, and TNF- α produced in the culture supernatant was measured by ELISA. Experiments were conducted three times with biological duplicates. (C) TNF- α concentration in supernatants collected 3 h postinfection from THP-1 cells challenged with WT *S. pneumoniae*, Δ NanA mutant, or the Δ NanA mutant strain complemented with NanA or enzymatically inactive NanA expression plasmids. Experiments were performed twice with biological duplicates. (D) Addition of recombinant *Arthrobacter ureadaciens* sialidase (rAUS, 50 mU/ml) from *Arthrobacter ureadaciens* did not undo the reduction in the level of TNF- α stimulation observed with the Δ NanA mutant. (E) Purified recombinant NanA alone did not induce TNF- α release from unstimulated or lipoteichoic acid (LTA)-stimulated THP-1 monocytes. SPN, *S. pneumoniae*. (F) Permeabilization of hBMEC monolayers. Culture supernatants of THP-1 cells were collected 4 h after infection with WT *S. pneumoniae* or the Δ NanA mutant strain or mock infection, and then the permeability of hBMECs was assessed by measuring HRP passage following an 8-h incubation of hBMECs with the collected THP-1 supernatants. Experiments were done twice with biological triplicates. H.I., heat inactivated. (G) Neither WT *S. pneumoniae* bacterial culture supernatant nor purified recombinant NanA is sufficient to induce hBMEC permeability, indicating that NanA-mediated cytokine induction from THP-1 cells is the major inducing factor. VEGF = vascular endothelial growth factor, used at 200 ng/ml as a positive control. Statistical analysis was performed using Student's *t* test (B) or one-way analysis of variance (ANOVA) with Tukey's posttest (C and F). ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

Signaling pathways implicated in macrophage inflammatory activation by *S. pneumoniae* NanA. Bacterial infections are well known to activate pattern recognition pathways such as Toll-like receptors (TLRs) to initiate downstream signaling leading to mitogen-activated protein kinase (MAPK) and NF- κ B activation. We first analyzed the activation of ERK, Jun N-terminal protein

kinase (JNK), and p38 MAPKs by Western blotting using specific antibodies (Abs) recognizing the activated phosphorylated forms of each protein. We found that, while THP-1 cells infected with WT *S. pneumoniae* exhibited greater phosphorylation of ERK than those infected with the Δ NanA mutant (Fig. 3A), there was no detectable phosphorylation for p38 and JNK when subjected to

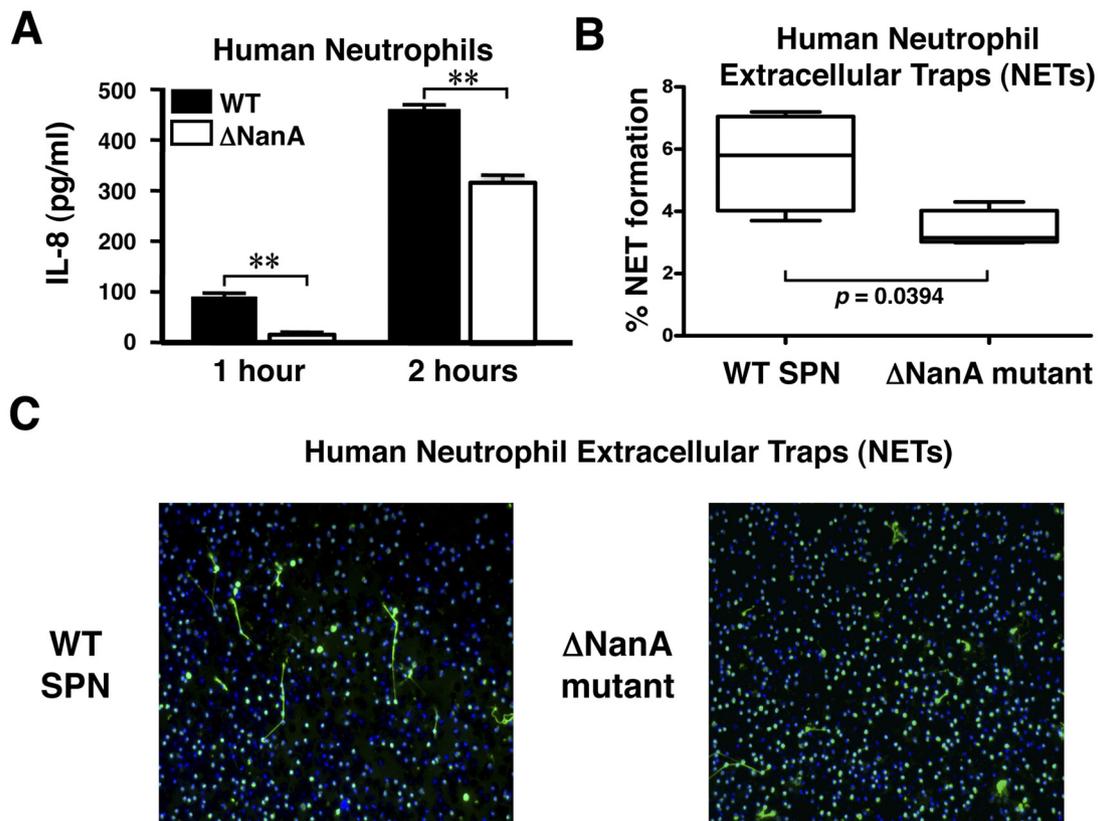


FIG 2 *S. pneumoniae* sialidase NanA increases neutrophil IL-8 secretion and extracellular trap (NET) formation. (A) Neutrophils were infected with WT *S. pneumoniae* or Δ NanA mutant (MOI = 10) for 1 or 2 h, and IL-8 released into the culture supernatant was measured by ELISA. Experiments were done twice with biological duplicates from two different donors. (B) Quantification of NETs after coinubation of neutrophils with WT *S. pneumoniae* or Δ NanA mutant at an MOI = 0.1 for 90 min. Cells were fixed and stained with primary rabbit anti-myeloperoxidase (anti-MPO) antibody followed by a secondary Alexa 488-conjugated goat anti-rabbit antibody to visualize NETs (green). DNA is stained with DAPI (blue). Representative immunofluorescence micrographs of NETs are shown in panel C, and quantitative results are shown in panel B. Statistics analysis was performed using Student's *t* test. **, $P < 0.01$.

the same stimulation (data not shown). In addition, infection of monocytes with WT *S. pneumoniae* led to accelerated degradation of $\text{I}\kappa\text{B}$ compared to infection with the Δ NanA mutant, indicating liberation of NF- κB for nuclear translocation and inflammatory cytokine gene transcription (Fig. 3A). Increased ERK phosphorylation and $\text{I}\kappa\text{B}$ degradation in response to *S. pneumoniae* sialidase activity are in accordance with the enhanced secretion of inflammatory cytokines in the *S. pneumoniae*-infected THP-1 cells (Fig. 1B and 1C) and with earlier published findings in which exogenous addition of a sialidase enzyme enhanced reactivity of phytohemagglutinin-treated lymphocytes (37) or ERK phosphorylation and cytokine production in LPS-treated monocytes (38).

CD33rSiglecs have been shown to downregulate both innate and acquired immune responses, likely via cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIMs) that recruit SHP family proteases to suppress tyrosine kinase-dependent signals (3, 39). Siglec-5 and Siglec-9 are the predominant CD33rSiglecs expressed on monocytes and neutrophils. Since THP-1 cells have been shown to express Siglec-5 but possess no detectable mRNA for Siglec-9 (40), we immunoprecipitated Siglec-5 to analyze SHP-2 recruitment status following WT *S. pneumoniae* or Δ NanA mutant infection. As shown in Fig. 3A, surface desialylation by WT *S. pneumoniae* infection reduced SHP-2 recruitment to Siglec-5 compared to results obtained with

Δ NanA mutant-infected monocytes. Temporally, diminished SHP-2 recruitment occurred earlier (30 min to 1 h postinfection) than ERK activation (1 to 2 h postinfection). This observation suggested that decreased inhibitory signals transduced from Siglec-5/SHP after surface desialylation to uncap Siglec receptors interaction could affect the activation status of the infected monocytes. We used RNA interference to silence the cell surface expression of Siglec-5 on THP-1 cells and confirmed knockdown efficiency by fluorescence-activated cell sorter (FACS) analysis. As shown in Fig. 3B, THP-1 cells infected with Siglec-5 short hairpin RNA (shRNA) lentivirus showed very little or no Siglec-5 staining compared to parental THP-1 cells and THP-1 cells infected with a control lentivirus. Supernatants from control and Siglec-5 knockdown THP-1 cells were then collected after infection with WT *S. pneumoniae* or Δ NanA mutant for TNF- α quantitation. As seen in parental THP-1 cells (Fig. 1B), *S. pneumoniae* infection induced more TNF- α secretion in control lentivirus-infected cells than did infection with the Δ NanA mutant (Fig. 3C). Although WT *S. pneumoniae* still triggered more TNF- α secretion than the Δ NanA mutant in Siglec-5 knockdown cells, the difference was not as pronounced as that observed in control cells. Further, the amount of TNF- α secretion in response to Δ NanA mutant infection was increased in the Siglec-5 knockdown THP-1 cells (Fig. 3C). Our findings suggested that NanA-mediated disruption of the Siglec-

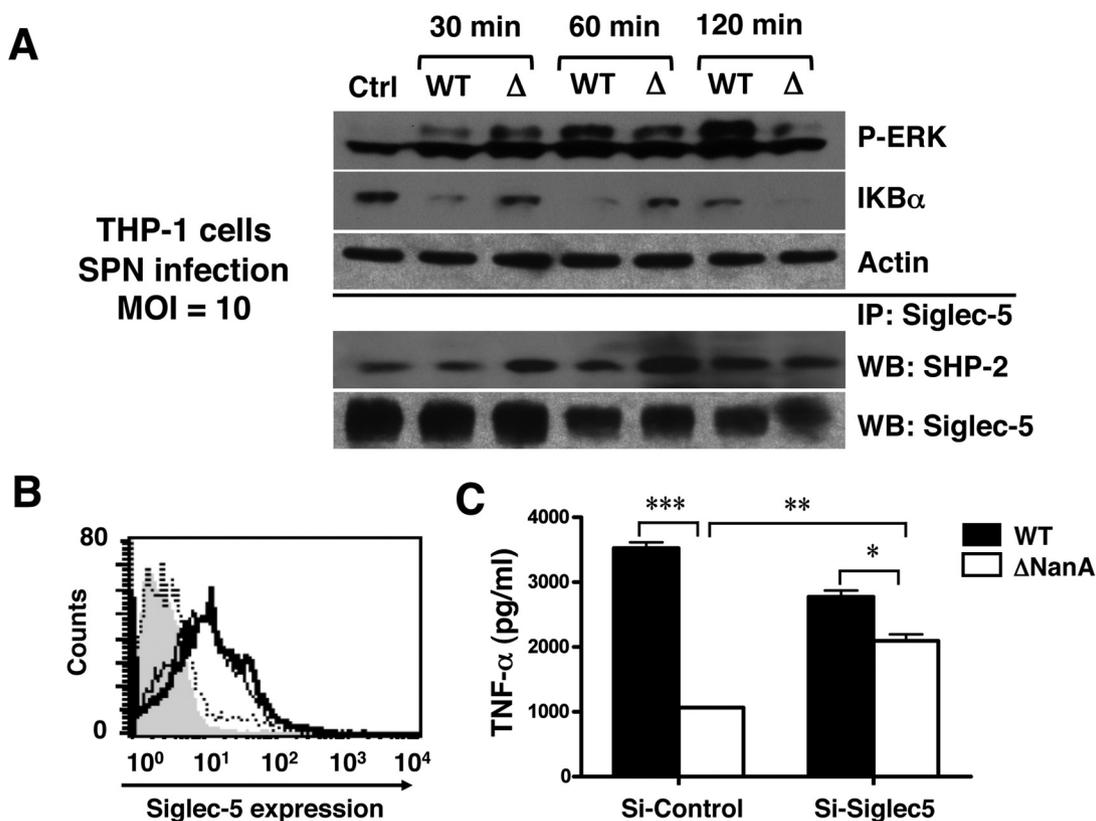


FIG 3 Signaling pathways implicated in macrophage inflammatory activation by *S. pneumoniae* NanA. (A) THP-1 cells were infected with WT *S. pneumoniae* or Δ NanA mutant at an MOI = 10. At the indicated times, cell lysates were analyzed by immunoblotting for ERK1/2 phosphorylation and I κ B degradation. SHP-2 recruitment to Siglec-5 was revealed by immunoprecipitating cell lysates with anti-Siglec-5 Ab, followed by probing with anti-SHP-2 Ab. (B) Knockdown of Siglec-5 expression by RNA interference. THP-1 cells were infected with lentiviruses carrying control shRNA (thin line) or Siglec-5 targeting shRNA (dashed line), and the knockdown efficiency was determined by FACS analysis with APC-conjugated anti-Siglec-5 MAb to measure cell surface Siglec-5 expression. THP-1 cells stained with APC-conjugated isotype MAb (solid gray) and anti-Siglec-5 MAb (thick line) served as negative and positive controls, respectively. (C) Control or Siglec-5 knockdown THP-1 cells were infected with WT *S. pneumoniae* or Δ NanA mutant at MOI = 10 for 3 h, and the culture supernatants were collected to determine TNF- α concentrations. Experiments were conducted twice with biological duplicates. Statistical analysis was performed by one-way ANOVA with Tukey's posttest. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

cis ligand interactions by surface desialylation may reduce Siglec-mediated inhibitory signals and contribute to the phenomenon of increased macrophage proinflammatory cytokine release.

***S. pneumoniae* NanA expression increases production of multiple inflammatory cytokines in a mouse intranasal infection model.** Previous work in a chinchilla otitis model showed that infection with WT *S. pneumoniae* but not a NanA-deficient mutant strongly decreased *Sambucus nigra* agglutinin labeling, indicating loss of cell surface sialic acid residues and resulting in exposure of underlying galactose residues (41). We examined whether infectious challenge with WT *S. pneumoniae* versus the Δ NanA mutant would alter proinflammatory cytokine responses *in vivo* using a murine intranasal challenge model of pneumonia. Significantly higher levels of TNF- α (6 h postchallenge) and IL-6 (20 h postchallenge) were present in the bronchoalveolar lavage (BAL) fluid of WT *S. pneumoniae*-infected animals compared to BAL fluid recovered from Δ NanA mutant-infected mice (Fig. 4A and B). At the 20 h postinfection time point, significantly increased levels of IL-6 and IL-1 β were identified in homogenates of lung tissue (Fig. 4C and D). The differences in cytokine production in BAL fluid and lungs were not due to different numbers of macrophages and neutrophils, because similar cell counts ($P =$

0.1649) were obtained with the BAL fluid recovered from WT *S. pneumoniae* and Δ NanA mutant-infected animals (Fig. 4E). Interestingly, fewer WT *S. pneumoniae* bacteria than Δ NanA mutant bacteria were recovered from BAL fluids (Fig. 4F), suggesting that a greater inflammatory response or NET induction elicited by NanA sialidase activity increased bacterial clearance during the short-term-infection period.

DISCUSSION

In the present report, we demonstrate that *S. pneumoniae* NanA augments proinflammatory cytokine production by human THP-1 cells and neutrophils during *S. pneumoniae* infection *in vitro*. This novel function of NanA-mediated desialylation may derive from unmasking of an inhibitory CD33rSiglec(s) from *cis* ligands and was corroborated by accelerated I κ B degradation, enhanced ERK phosphorylation, and reduced SHP-2 recruitment to Siglec-5.

The mammalian host protects itself from infection through rapid recognition of pathogens and activation of innate immune responses; however, unregulated activation can produce detrimental consequences of systemic inflammation, multiorgan failure, and disseminated intravascular coagulation (DIC) (42, 43).

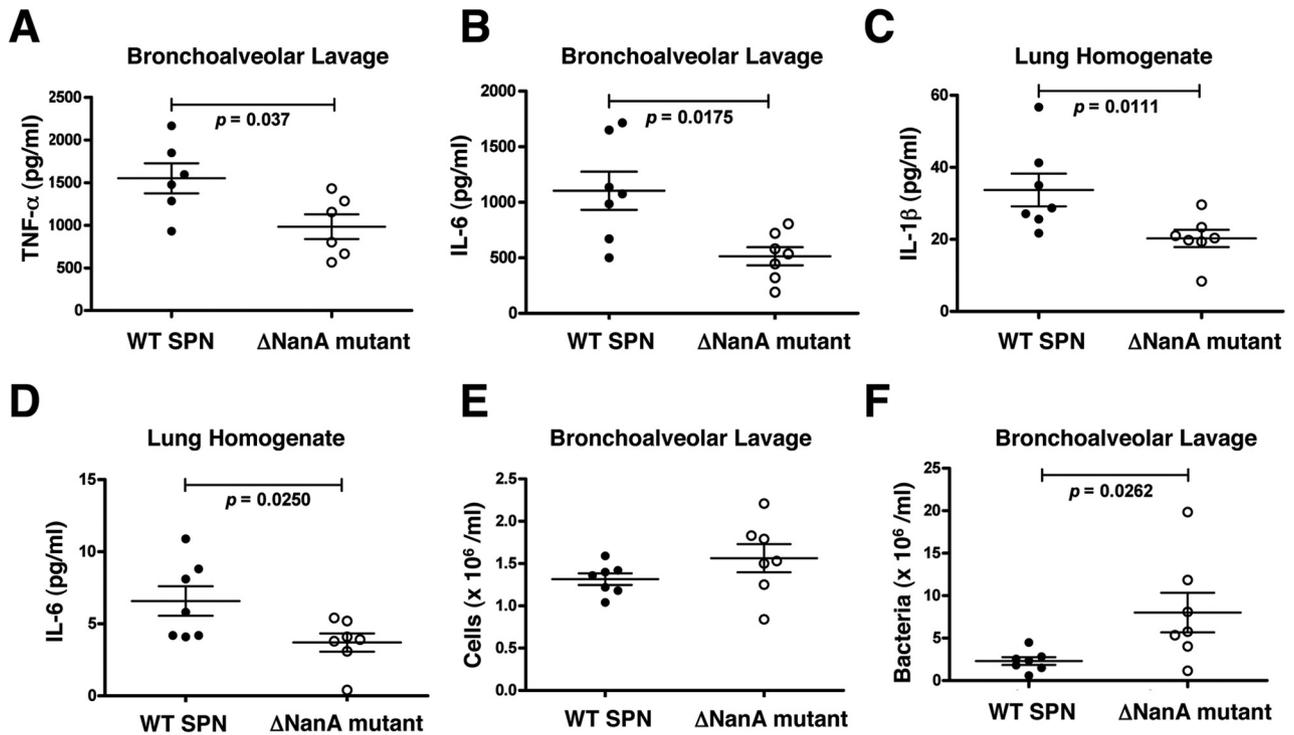


FIG 4 *S. pneumoniae* NanA expression increases production of multiple inflammatory cytokines in a mouse intranasal infection model. Mice were infected intranasally with 1×10^7 CFU of WT *S. pneumoniae* or Δ NanA mutant, and cytokine levels in cell-free BAL fluid or lung homogenates were measured by ELISA. (A) TNF- α in BAL fluid at 6 h; (B) IL-6 in BAL fluid at 20 h; (C) IL-1 β in lung homogenates at 20 h; (D) IL-6 in lung homogenates at 20 h. (E) Recruitment of inflammatory cells from the circulation to BAL fluid was determined using a Beckman particle counter 20 h postinfection. (F) Bacterial load (CFU) in BAL fluids of mice 20 h postinfection. Differences between the 2 groups were analyzed by Mann-Whitney test.

Therefore, the innate immune system needs to be tightly controlled, and it is widely accepted that receptors containing immunoreceptor tyrosine-based inhibitory motifs (ITIMs) can be employed to switch off cellular responses through recruitment of phosphotyrosine phosphatases (PTPs) such as SHP-1 and SHP-2 to decrease tyrosine phosphorylation. Moreover, the engagement of inhibitory ITIM-containing receptors also blocks activation signals originating from receptors associated with immunoreceptor tyrosine-based activating motifs (ITAMs) (44, 45). Given the ubiquitous expression of sialic acids on mammalian cell surfaces and the prominence of cognate ITIM-bearing CD33rSiglecs on immune cells, Siglecs are speculated to control the activation threshold of immune cells (3, 5), using sialic acids as “self-associated molecular patterns” (9). Our findings provide new evidence to support the hypothesis that unmasking Siglecs from *cis* ligands can increase immune cell inflammatory responses.

We observed more SHP-2 recruitment to Siglec-5 in THP-1 monocytes after infection with the Δ NanA mutant compared to those infected with the WT *S. pneumoniae* parent strain. This finding suggests that reduced Siglec-mediated inhibitory signals are involved in the NanA-dependent proinflammatory cytokine stimulation phenotype. In addition, knockdown of the expression of Siglec-5 in the THP-1 cells reduced differential cytokine induction elicited by WT *S. pneumoniae* versus the Δ NanA mutant. This finding further supported the hypothesis that unmasking of Siglec-5 by NanA is critical to enhance proinflammatory cytokine production during *S. pneumoniae* infection. THP-1 cells have no detectable mRNA of Siglec-9 (40); however, knockdown of the

Siglec-5 expression still did not completely abolish the NanA-dependent proinflammatory cytokine induction increases. Although the NanA mutant in *S. pneumoniae* strain D39 has negligible residual activity, as shown by *in vitro* sialidase assays (32, 46), an indispensable secondary role of *S. pneumoniae* sialidases such as NanB cannot be summarily excluded (47). In addition, mammalian Neu1 sialidase was shown to form a complex with Toll-like receptor-2 (TLR2), TLR3, and TLR4 and facilitate TLR4 clustering, MyD88/TLR4 complex formation, and subsequent NF- κ B activation upon LPS engagement (48, 49). Indeed, purified *S. pneumoniae* NanA and *Trypanosoma cruzi* trans-sialidase were demonstrated to activate NF- κ B signaling pathways in the absence of TLR ligands (48). Therefore, it is possible that, in addition to inhibitory Siglec unmasking, TLR2/TLR6, a receptor complex for Gram-positive bacterial recognition, could also be desialylated by endogenous Neu1 or by NanA itself upon *S. pneumoniae* infection, facilitating its dimerization and downstream signaling activation.

NanA-mediated desialylation affects numerous glycoconjugates (glycoproteins and glycolipids) on the host cell surface, since their structures are often capped by terminal sialic acids. Furthermore, such desialylation can simultaneously expose underlying *N*-acetylglucosamine (Gal β 1-4GlcNAc) ligands for galectins. Galectins can bind and translate this glycan-encoded information into immune cell activation, differentiation, and homeostatic programs. These lectins appear to function by forming ordered galectin-glycan lattices on the cell surface, leading to immunoregulatory activities (50, 51). For example, galectin-3-mediated ligand

clustering triggered neutrophils to phagocytose, produce reactive oxygen species, release proteases, and secrete IL-8 (52, 53). On the other hand, galectin-1 inhibited nitric oxide synthesis and increased the arginase activity of macrophages (54). The immunomodulatory effects of galectins may be integrated into the cumulative effects on proinflammatory cytokine secretion observed after NanA-mediated desialylation. However, due to the short-duration experiments performed on THP-1 cells and neutrophils, the endogenous concentration of galectins in our assay systems should not be as high as those reported from studies in the previous literature, in which purified exogenous galectins were added into immune cells. By knocking down the expression of Siglec-5, we demonstrated that Siglec-cis ligand interactions are themselves a critical control element in the activation status of immune cells.

All *S. pneumoniae* clinical isolates express the surface-anchored NanA (55, 56), which can cleave terminal sialic acid residues α 2-3 or α 2-6 linked to galactose, mirroring the ligand preferences of several CD33rSiglecs. NanA cleaves α 2-3- and α 2-6-linked substrates with equal levels of efficiency and exhibits more than 10-fold-greater overall sialidase activity than NanB, an enzyme that exhibits a 5-fold preference for α 2-3 over α 2-6 linkages (57). Prior work has established that NanA plays multiple roles in *S. pneumoniae* pathogenesis, including modification of the nasopharyngeal epithelial surface to reveal adherence receptors (58–61), biofilm formation (62), provision of free carbohydrates for bacterial metabolism (31, 63, 64), desialylation of the cell surfaces of niche competitors such as *Neisseria meningitidis* and *Haemophilus influenzae* (65), modification of platelets to promote their clearance by the hepatic Ashwell receptor (66), and blood-brain barrier endothelial cell invasion (32, 67). Our results add stimulation of leukocyte proinflammatory responses to the list of phenotypic changes in the host associated with this multifactorial *S. pneumoniae* virulence factor.

We also demonstrated that WT *S. pneumoniae* was more potent than the Δ NanA mutant in stimulating proinflammatory cytokine production in BAL fluid or lung homogenates following murine intranasal challenge, findings associated with recovery of fewer WT *S. pneumoniae* bacteria than Δ NanA mutant bacteria from BAL fluid culture in the short-term-infection model. A recent report demonstrated that WT *S. pneumoniae* induced greater inflammatory cytokine secretion and higher mortality than a NanA⁻ NanB⁻ double mutant, even though similar CFU counts of the two strains were recovered in the bloodstream following intraperitoneal infection (68). Together, these data suggest that augmented cytokine responses and neutrophil activation upon Siglec-mediated “detection” of *S. pneumoniae* or of other sialidase-expressing pathogens could boost the initial localized innate immune response; however, the same processes during severe infections could provoke widespread dysregulation of inhibitory Siglec function and uncontrolled systemic inflammatory reactions that contribute to tissue injury, shock, or death. In this regard, total sialidase activity measured by fluorescent assay was significantly higher in blood collected from septic patients than in blood from nonseptic patients or healthy controls (69).

Elevated concentrations of free sialic acid in cerebrospinal fluid (CSF) concentrations were detected in 17 of 35 patients with acute *S. pneumoniae* meningitis, while patients with *Haemophilus influenzae* or *Neisseria meningitidis* meningitis had relatively normal free CSF sialic acid levels (70). Previous work has shown that NanA contributes to *S. pneumoniae* blood-brain barrier invasion

and meningeal inflammation in the murine meningitis model (32, 67). Here we found that NanA-mediated proinflammatory cytokine release from macrophages can increase the permeability of hBMECs, which may be a further contributing mechanism to bacterial and neutrophil influx into the central nervous system (CNS) from the bloodstream.

Secondary *S. pneumoniae* pneumonia is a major complication of influenza pneumonia and accounts for excess mortality during influenza epidemics (71). In a mouse model of *S. pneumoniae*, after influenza virus pulmonary infection, strikingly elevated levels of proinflammatory cytokines, including TNF- α , IL-6, and IL-1 β , coupled with massive neutrophil influx, were observed in the lungs (72). A potential role for influenza virus neuraminidase in this lethal synergism has been proposed to involve exposure of binding receptors for *S. pneumoniae* on respiratory epithelium (73). Our present data suggest that, in addition to this mechanism, the viral and bacterial neuraminidases could synergize during severe infection to cause widespread cell surface desialylation and trigger overexuberant pulmonary and systemic inflammatory responses.

In conclusion, we present the observation using WT and isogenic mutant *S. pneumoniae* bacteria that the NanA sialidase can enhance the inflammatory response of immune cells. The unmasking of inhibitory CD33rSiglecs to remove inhibition of MAPK and NF- κ B signaling represents a potential mechanism contributing to this phenomenon. Further analysis of sialic acid and Siglec receptor interactions during host-pathogen encounters could provide novel targets for therapeutic intervention to modify infectious disease outcome.

MATERIALS AND METHODS

Reagents. Purified sialidase from *Arthrobacter ureafaciens* and *pneumoniae* was purchased from EY Laboratories (San Mateo, CA) and QA-Bio (Palm Desert, CA), respectively. Lipoteichoic acid was from InvivoGen (San Diego, CA), and VEGF₁₆₅ was from PeproTech (Rocky Hill, NJ).

Bacterial strains and growth conditions. *S. pneumoniae* serotype 2 strain D39 (NCTC 7466) and its isogenic Δ NanA mutant were used for these experiments. The Δ NanA mutant was constructed by nonpolar allelic replacement mutagenesis of the *nanA* gene as previously described (46). Strains in which the Δ NanA mutant was complemented with an expression vector for the wild-type enzyme (pNanA) or a catalytically inactive mutant enzyme (pNanA Δ Enz) were previously validated by flow cytometry for surface expression of the protein (both plasmids) and fluorescent assay. For restoration of sialidase activity (pNanA only) (32), *S. pneumoniae* cultures were grown in Todd-Hewitt broth with 2% yeast extract (THY media) THY with chloramphenicol (2 μ g/ml) was used to propagate the complemented strains. Freshly cultured bacteria from frozen aliquots were grown at 37°C in 5% CO₂ and THY broth to the log phase (optical density at 600 nm [OD₆₀₀] = 0.4) for all infection experiments.

Cell culture and *in vitro* *S. pneumoniae* infection. THP-1 cells a human acute monocytic leukemia cell line were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). Human neutrophils were isolated from healthy donors by the use of the PolymorphPrep system (Axis-Shield, Fresenius, Waltham, MA) and were suspended in serum-free RPMI 1640 medium for experiments. Cells from the well-characterized simian virus 40 (SV40) large T antigen-immortalized human brain endothelial cell line (hBMEC), originally obtained from Kwang Sik Kim (Johns Hopkins University, Baltimore, MD), was maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS, 10% NuSerum (BD Biosciences), and 1% nonessential amino acids. THP-1 cells or neutrophils were resuspended in culture medium at the cell density of 1×10^7 /ml. WT *S. pneumoniae* and the Δ NanA mutant

were grown to the logarithmic phase, washed once with phosphate-buffered saline (PBS), and resuspended in culture medium to an $OD_{600} = 0.1$ ($\sim 10^8$ CFU/ml) and then further diluted to the desired inoculum. Bacteria were added to 5×10^6 THP-1 cells or neutrophils in 2-ml siliconized microtubes at a multiplicity of infection (MOI) of 10, 3, or 1 as specified. The mixture of leukocytes and bacteria was incubated at 37°C with rotation, penicillin (5 μ g/ml) and gentamicin (100 μ g/ml) were added 30 min after infection to prevent bacterial overgrowth, and the cells or supernatants were harvested at the indicated time points for analysis.

FACS analysis of cell surface desialylation. THP-1 cells were infected with WT *S. pneumoniae* or Δ NanA mutant at an MOI of 10, 3, or 1 for 3 h as described above. After infection, cells were washed twice with PBS and stained with fluorescein isothiocyanate (FITC)-conjugated *E. cristagalli* agglutinin lectin (Vector Laboratories, Burlingame, CA) for 30 min at 4°C. THP-1 cells were then washed twice and subjected to FACSCalibur flow cytometry (BD Biosciences) to analyze the exposed Gal β 1-4GlcNAc β 1 units.

RNA interference. Human Siglec-5 lentiviral shRNA construct (TRCN0000062525) was purchased from Open Biosystems. The targeting viruses were produced by cotransfection of 293T cells with the shRNA plasmid and packaging vectors (Open Biosystems) according to the vendor's instructions. Knockdown efficiency was determined by staining the surface expression of Siglec-5 by the use of allophycocyanin (APC)-conjugated anti-Siglec-5 monoclonal antibodies (MAbs) (BD Biosciences).

Mouse intranasal infection and sample collection. All animal experiments were approved by the Committee on the Use and Care of Animals, University of California, San Diego, and performed using accepted veterinary standards. Mice were lightly anesthetized by intraperitoneal injection of ketamine and xylazine, and 50 μ l of PBS containing 1×10^7 *S. pneumoniae* CFU was then administered into the nostrils of the mice. The inoculum dose was confirmed by CFU counts on THY agar plates. Infected animals were sacrificed 6 h (6 mice in each group) or 20 h (7 mice in each group) postinfection. Blood was collected via terminal cardiac puncture. For bronchoalveolar lavage (BAL) fluid collection, the trachea was exposed and 0.8 ml of PBS (without calcium or anticoagulant) was injected twice using an 18-gauge, 1 and 1/2-in.-long needle connected to a 1 ml syringe. A 25- μ l volume of BAL fluid was serially diluted and plated on THY plates to enumerate CFU. The rest of the BAL fluid was centrifuged at 1,500 rpm for 10 min and the supernatant frozen at -80°C for cytokine analysis. Cells were analyzed using a Z1 particle counter (Beckman Coulter) to determine the total cell count in the BAL fluid. The right lung was excised and placed into sterile preweighed 2-ml screw-cap microtubes containing 1 ml of sterile PBS and 1-mm-diameter zirconia/silica beads (BioSpec Products). After the tubes were weighed to determine lung weight, lung tissues were disrupted by a 1-min homogenization burst using a Mini-BeadBeater (BioSpec Products), followed by centrifugation at 12,000 rpm to collect supernatants for cytokine analysis.

Cytokine detection. The concentrations of cytokines in THP-1 supernatants collected at various time points postinfection were quantified for IL-6, IL-8, and TNF- α using enzyme-linked immunosorbent assays (ELISA) according to the instructions of the manufacturer (R&D Systems, Minneapolis, MN). Mouse ELISA kits (TNF- α and IL-6 from R&D and IL-1 β from BD Biosciences) were used to detect cytokines in BAL fluids and lung homogenates of infected animals.

Human brain microvascular endothelial cell (hBMEC) permeability assay. hBMECs (2×10^4) were seeded on collagen-coated Transwell inserts (Costar) (pore size, 3 μ m) for 3 to 4 days to attain confluence, which was verified by direct visualization and resistance to Evans Blue dye leakage in control wells. At the beginning of the experiment, the medium in the upper compartment was carefully replaced with 150 μ l of supernatant collected from uninfected, WT *S. pneumoniae*-infected, or Δ NanA mutant-infected THP-1 cells, while the lower compartment was replenished with fresh medium. After 8 h of incubation at 37°C with 5% CO₂, 10 μ l of horseradish peroxidase (HRP) (50 μ g/ml) was added to the upper

chamber for an additional 30 min. A 10- μ l volume of the medium from the lower chamber was diluted in 190 μ l of PBS, followed by addition of TMB substrate (BD Biosciences) for 20 min; the reaction was stopped by the use of 2N H₂SO₄, and results were read at OD₄₅₀.

Neutrophil extracellular trap (NET) visualization and quantification. Neutrophils (2.5×10^5) were seeded on 48-well plates, infected with WT *S. pneumoniae* or Δ NanA mutant at an MOI = 0.1, centrifuged at 1,600 rpm for 5 min, and incubated for 90 min at 37°C with 5% CO₂. To visualize NETs, neutrophils were incubated with rabbit polyclonal antibodies against myeloperoxidase (Dako), followed by staining with 4',6'-diamidino-2-phenylindole (DAPI) and Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen) as previously described (74). Images were recorded using a Zeiss Axiovert microscope. The total amount of neutrophils and the amount of neutrophils releasing NETs per field of view were counted in 4 individual images per sample. The same investigator (Y.-C. Chang) performed the experiment and the quantification.

Western blot analysis. THP-1 cells were lysed in buffer (50 mM Tris [pH 8], 150 mM NaCl, 1% NP-40) containing protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Santa Cruz Biotechnology). Cell lysates were then separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was probed with the anti-phospho-p44/42 MAPK (T202/Y204; Cell Signaling Technology), anti-I κ B (Cell Signaling Technology), or anti-actin (Sigma) Abs, followed by appropriate HRP-conjugated secondary Abs (Bio-Rad) and ECL reagent (Thermo Scientific). Siglec-5 immunoprecipitation was performed as previously described (75). Briefly, 500 μ g of cell lysate was immunoprecipitated with 1A5 anti-human Siglec-5 MAb (gift from P. Crocker, University of Dundee, Dundee, Scotland, United Kingdom) and protein G Sepharose beads (BD Biosciences). The Western blotting method described above was used to probe samples with rabbit anti-SHP-2 Abs (Santa Cruz Biotechnology).

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