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Preserving Sialic Acid-dependent Pattern Recognition by CD24-Siglec G Interaction for Therapy of Polybacterial Sepsis

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Abstract

Control of inflammation is critical for therapy of infectious diseases. Pathogen-associated and/or danger-associated molecular patterns (PAMPs and DAMPs, respectively) are the two major inducers of inflammation. Because the CD24-Siglec G/10 interactions selectively repress inflammatory response to DAMPs, microbial disruption of the negative regulation would provide a general mechanism to exacerbate inflammation. Here we show that the sialic acid-based pattern recognitions of CD24 by Siglec G/10 are targeted by sialidases in polybacterial sepsis. Sialidase inhibitors protect mice against sepsis by a *CD24-Siglecg*-dependent mechanism, whereas a targeted mutation of either *CD24* or *Siglecg* exacerbates sepsis. Bacterial sialidase and host *CD24* and *Siglecg* genes interact to determine pathogen virulence. Our data demonstrate a critical role for disrupting sialic acid-based pattern recognitions in microbial virulence and suggest a therapeutic approach to dampen harmful inflammatory response during infection.

Introduction

The current paradigm of microbial virulence is largely pathogen-centric. Among the key considerations are tissue tropism, replication/growth rates and the cytopathic effect of the microbes. The impact of the host's immune system is also largely viewed from the prism of

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its impact on clearance of pathogens. However, it is increasingly clear that inflammation is a critical determinant for pathogen virulence. For example, pigtailed macaques (*Macaca nemestrina*) and African green monkeys (*Chlorocebus sabaeus*) support viral replication equally well, yet, AIDS-like syndrome can be observed in *Macaca nemestrina* but not in the *Chlorocebus sabaeus*. It has been suggested that the difference is due to the magnitude of inflammatory responses [1]. Another notable example is sepsis. Despite availability of antibiotics, the mortality and hospitalization of severe sepsis increased rapidly between 1993 and 2003, causing approximately 200,000 annual deaths in the US alone [2]. Our inability to control the disease highlights a major gap in understanding the root-cause of inflammation associated with infections.

Among the best defined causes of inflammation are the pathogen-associated molecular patterns (PAMPs) that interact with pattern-recognition receptors, such as toll [3] or toll-like receptors (TLR) [4] and Nod-like receptors (NLR) (reviewed by [5, 6]. Since PAMPs should be cleared soon after the bacteria are eliminated by antibiotics, the fact that antibiotics cannot cure sepsis suggests that PAMPs are not the only root cause of sepsis. Another source of inflammation is the danger (or damage)-associated molecular patterns (DAMPs) [7], the conserved intracellular structures exposed after cell deaths, including necrosis or secondary necrosis following apoptosis [8]. The role of high mobility globulin B1 (HMGB1) in the pathogenesis of sepsis [9] demonstrates a critical role of host recognition of DAMPs. However, while DAMPs released during infection may also trigger inflammation through TLR and/or NLR receptors [10-12], our recent studies have indicated that host response to DAMPs is negatively regulated by CD24-Siglec G/10 interactions [13]. As a result, inflammation caused by DAMPs is likely limited unless the CD24-Siglec G (mouse)/10 (human) interactions are disrupted. Therefore, it is intriguing that the CD24-Siglec G/10 interaction may be disabled during infection.

Siglec G/10 is a member of Siglecs, immunoglobulin super family lectins with a defining feature of recognizing sialic acid-containing structures [14]. Human Siglec 10 is the functional homologue of mouse Siglec G and it binds both mouse and human CD24 [13]. Since most of the Siglec family members contain ITIM- or ITIM-like motifs and many are known to be associated with SHP-1 and/or SHP-2[14], it has been suggested that the Siglec family member may serve as a negative regulatory pathway for cellular activation. In support of a negative regulatory activity of this pathway in immune recognition, the sialic acid-based pattern recognition has recently been shown to negatively regulate both antibody production [15-17] and neutrophil activation [18]. However, the possibility that sialic acid-based pattern recognition may be disrupted by sialidases during infection to exacerbate pathogen virulence has never been tested. Since many pathogens encode sialidases as their virulence factor, we tested the hypothesis using the polybacterial sepsis model.

Results

Targeted mutation of CD24 and Siglec G exacerbate the polybacterial sepsis

We used the cecal ligation and puncture (CLP) [19] as the basic model to evaluate the potential contribution of CD24-Siglec G interaction in lethal sepsis. We employed two different procedures with different mortalities in WT mice in the current study. First, to

mimic the clinical setting, we treated mice with antibiotics after CLP. The use of antibiotics may help to demonstrate that CD24-Siglec G interaction regulates virulence of sepsis even when bacterial growth is inhibited. A 23G3/4 needle was used for puncture so that the majority of the WT mice will survive the procedure. The antibiotic was highly effective as no bacteria were isolated in the blood samples from antibiotic-treated mice (data not shown). As shown in Fig. 1a, while 70% of WT mice survived the entire period of observation, the overwhelming majority of the mice with targeted mutation of either *CD24* or *Siglecg* died within 48 hours. Corresponding to the increased mortality in the mutant mice, the levels of IL-6, MCP-1 and TNFα was sharply elevated (Fig. 1b. Apart from IL-6 and TNFα, the level of C5a was also somewhat elevated (supplemental Fig. S1a). However, the levels of MIF were not elevated in the $CD24^{-/-}$ and $Siglecg^{-/-}$ mice (Supplemental Fig. S1b).

To substantiate this observation, we tested the impact of the targeted mutations in a more severe model of sepsis that involves a larger needle for puncture. In order to reveal the potential effect of mutations on bacterial burden in the blood, the mice received no antibiotic treatment. As shown in Fig. 1c, targeted mutations resulted in significant acceleration of onset and increased mortality following CLP. However, the bacterial burden in the blood was unaffected by these mutations (Fig. 1d). Since most bacteria in the cecum are obligate anaerobes that cannot be detected, our assay did not address whether bacterial growth in hypoxic environment may be affected. The increased mortality in the mice with mutation of either *CD24* or *Siglecg* corresponds to a significant increase of inflammatory cytokines (Fig. 1e). In fact, the inflammatory cytokines were significantly elevated in WT mice only after 24 hours (Fig. 1f). Nevertheless, the magnitudes of cytokine elevation do not explain the overall increase of mortality in the more severe model. The presence of more live bacteria may have also contributed to the increased virulence. Compared to WT counterparts, the lung, kidney and liver of $CD24^{-/-}$ and $Siglecg^{-/-}$ mice showed severe hemorrhage and venous congestion. The kidney sections showed acute tubular necrosis. These findings suggested hypotension and acute multi-organ failure (Fig. 1g)

We produced transgenic mice expressing CD24 under the control of CD11c promoter [22, 23]. As shown in Fig. 2a, upon crossing to the $CD24^{-/-}$ background, $CD11c^{+}$ MHC class II^{+} cells expressed high levels of CD24, which is comparable to WT. While detectable, levels of CD24 found in the MHC class II^{+} $CD11c^{-}$ cells were about 50-fold lower than WT counterparts. The transgenic mice had a better survival rate (Fig. 2b) although the burden of live bacteria in the blood was unaffected by the transgene (Fig. 2c). Correspond to better survival, the transgenic mice produced less inflammatory cytokines than the CD24-deficient littermates (Fig. 2b, 2d). Therefore, CD24 expressed predominantly on $CD11c^{+}$ cells promoted survival and suppressed inflammation without affecting bacterial burden.

CD24-Siglec 10 interaction in vitro depends on sialic acid on CD24

The impact of genetic disruption of the CD24-Siglec G interaction indicates that this pathway negatively regulates inflammation during sepsis and thus raises an intriguing possibility that this interaction may be targeted to exacerbate sepsis. Because many pathogenic bacteria encode sialidases as a virulence factor [24], and because sialic acid-based pattern recognition is a cardinal feature of Siglecs [14], we considered the possibility that

bacterial sialidases may exacerbate sepsis by CD24 desialyation. To determine whether the CD24-Siglec 10 interaction may be susceptible to bacterial sialidases, we treated the CD24Fc with recombinant sialidases of three different bacteria, *S. pneumoniae*, *C. perfringens*, and *V. cholerae*. The interaction between CD24 and Siglec 10 fusion proteins were measured by co-immunoprecipitation and a solid-phase binding assay. All sialidases abolished CD24-Siglec 10 interaction (Fig. 3a), while the treatment only partially reduced reactivity to anti-CD24 mAb. Consistent with reported specificity of Siglec 10Fc [25], the CD24-Siglec 10 interaction was inhibited by either α2–3 or α2–6-linked N-acetyl lactosamine, although the α2–6 sialoside was more potent (Fig. 3b). We used sialidase-desialylated CD24Fc with and without additional sialyltransferase-resialylation (supplemental Fig. S2) to substantiate these observations. As shown in Fig. 3c and 3d, either α2–3 or α2–6 resialylation was sufficient to restore CD24-Siglec 10 interaction, although α2-6 resialylated CD24Fc exhibited better binding. Nevertheless, since the α2–6 sialylation is less abundant than the α2-3 sialylation (Supplemental fig. S2), untreated CD24Fc required α2-3 sialylation for binding to Siglec 10. This is consistent with the fact that sialidase specific for α2-3-linked sialic acid disrupted the CD24Fc-Siglec 10-Fc interaction (Fig. 3a). Moreover, since more than 20-fold excess of desialylated CD24 failed to give significant binding to plate-coated Siglec 10Fc (Fig. 3d), apparent variations in the amounts of CD24Fc recovered after enzymatic treatment (Fig. 3a), as judged by reactivity to anti-CD24, do not explain the almost complete lack of CD24-Siglec 10 interaction after sialidase treatment (Fig. 3a, d). In combination, the data of sialidase digestion of CD24Fc and inhibition of CD24-Siglec 10 interaction by sialosides suggest that both α2–3 and α2–6-linked sialosides could interact with Siglec 10.

Desialyation of WT but not *CD24*$^{-/-}$ and *Siglecg*$^{-/-}$ DC increase their response to HMGB1 and heat-shock protein (HSP) 70

To measure the effect of desialyation on DC response to DAMPs, we treated DC from WT, *CD24*$^{-/-}$ and *Siglecg*$^{-/-}$ mice with sialidase and tested their response to HMGB1 and HSP70. As shown in Fig. 3e, desialyation of WT DC, but not that of *CD24*$^{-/-}$ and *Siglecg*$^{-/-}$ DC significantly increased the production of inflammatory cytokines. Thus, even though sialidase used has a large array of substrates, the requirement for both *Siglecg* and *CD24* genes for its biological function provided genetic evidence that disruption of CD24-Siglec G interaction is responsible for the enhancement of cytokine production by sialidase. To test if desialyation affect CD24 binding to HMGB1, we used pull-down assay to measure the effect of desialylation on CD24Fc binding to HMGB1. As shown in Fig. 3f, desialylated CD24Fc barely reduced its binding to HMGB1. Thus, disruption of CD24-HMGB1 interaction is not responsible for enhanced cytokine production by DC.

Disruption of CD24-Siglec G/10 interaction in polybacterial sepsis

We then tested the potential role for sialidase in the CLP model. As shown in Fig. 4a, following CLP, a clear elevation of serum sialidase activity was observed. Sera from mice with sham surgery did not show a significant increase in sialidase activity. Moreover, since LPS treatment that induced a strong inflammatory response did not cause elevation of sera sialidase activity, inflammation alone was insufficient to cause release of sufficient sialidase. Since sera consist of large amounts of sialylated proteins that serve as substrates of sialidase,

the overall sialidase activity is likely underestimated. The bacteria released appear sufficient to cause sialidase increase as antibiotic-treated mice also show elevated sialidase level (Fig. S3). Regardless of mouse genotypes, the sera from CLP mice, but not those from sham-surgery control, disrupted CD24-Siglec 10 interaction (Fig. 4b).

In order to determine whether polybacterial sepsis ablates CD24-Siglec 10 interaction, we compared both total CD24 levels and Siglec 10-binding activity in the spleen cells in sham- or CLP-treated mice. Although the total level of CD24 was not reduced in the CLP group (Fig. 4c), a substantial reduction of Siglec 10-binding was observed by flow cytometry (Fig. 4d). Thus, sepsis disrupted CD24-binding to Siglec 10, the human homologue of mouse Siglec G.

To confirm that Siglec G binding to CD24 is also affected, we generated Siglec G-Fc fusion protein and used it in flow cytometry. As shown in Fig. 4e, f, a similar reduction in Siglec G-Fc binding to spleen cells of septic mice were observed. Importantly, double mutations (117Y>A; 120R>A) of the sialic acid-binding site in the Siglec G abrogated the binding to spleen cells (Fig. 4f). Therefore, the defective binding to Siglec G-Fc in CLP-treated mice was due to desialyation. Corresponding to reduced Siglec 10/G-binding activity, increased electrophoresis mobility was observed in CD24 on the spleen cells from the CLP mice (Fig. 4g). Correspondingly, significant reductions in binding of lectins that recognize either α2–3-, or α2–6-linked sialic acids in both total spleen cells (Fig. 4h) and gated DC subset (Fig. 4i) were also observed.

Therapeutic potential of targeting sialidase in polybacterial sepsis

To test the role of bacterial sialidases in pathogenesis of sepsis, we synthesized two sialidase inhibitors, 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid (Neu5Ac2en) and 2,3-dehydro-2-deoxy-*N*-glycolylneuraminic acid (NeuGc2en). We first tested their inhibition against sialidase activity in the mouse serum after CLP. As shown in Fig. 5a, while Neu5Ac2en had a minimal effect on the sialidase activity, a partial inhibition was observed for Neu5Gc2en. A combination of the two inhibitors completely inhibited the sialidase activity in the sera of septic mice. Importantly, the sialidase inhibitors significantly reduced the mortality of sepsis (Fig. 5b). To confirm that the sialidase inhibitors protect mice by preserving the integrity of CD24-Siglec G interaction, we tested their activity in mice with targeted mutation of *Siglecg* and *CD24*. As shown in Fig. 5b, no protection was observed if either gene was deleted. Therefore, the inhibitory effect of the sialidase inhibitors was dependent on both CD24 and Siglec G. Regardless of the mouse strains used, the inhibitors do not significantly reduce bacterial burden in the blood at either 12 hours (Fig. 5c, left panel) or 24 hours after CLP (Fig. 5c, right panel). Correspondingly, multiple inflammatory cytokines were reduced by the inhibitors (Fig. 5d). The dependence on the *CD24* and *Siglecg* genes demonstrates a specificity of the inhibitors and suggests that the protection is likely achieved by preserving Siglec G-CD24 interaction. Since sepsis patients often received antibiotic treatment, we also tested if the sialidase inhibitor works in conjunction with antibiotics. As shown in Fig. 5e, while antibiotic alone had little impact on survival, sialidase inhibitors substantially increased mouse survival. The effect of sialidase inhibitors in antibiotics-treated mice

further confirms that the impact of sialidase inhibitors is unlikely due to its potential inhibition of bacterial growth.

Bacterial sialidases disrupt CD24-Siglec G interaction to exacerbate sepsis

To elucidate the contribution of bacterial sialidases to sepsis, we performed polymerase chain reaction of peritoneal washes in CLP mice to detect bacterial sialidase genes. DNA contamination was ruled out as no signal was found in aliquots of RNA that were not converted to cDNA by reverse transcriptase. PCR products were confirmed by DNA sequencing. As summarized in Table S1, of the 8 sialidases tested, 4 were found expressed in the bacteria in peritoneal wash. Although sialidase proteins from these bacterial cannot be formally demonstrated due to lack of reagents, the broad expression of microbial sialidase genes strengthen the contention that bacterial sialidases are produced in the CLP model.

Since *S. pneumoniae* expresses extracellular sialidases [26] and is a common cause of sepsis [27], and since both genes encoding NanA and NanB sialidases were expressed in the peritoneal of the CLP mice (Table S1), we generated mutant bacteria lacking both *nanA* and *nanB* from the D39 strain (mD39) and tested their pathogenicity in WT and mutant mice. As shown in Fig. 6a, deletion of both *nanA* and *nanB* genes eliminated the sialidase activity in the bacterial culture. As expected, mice infected with WT *S. pneumoniae* exhibited higher sialidase activity in the sera than those infected with the mutant (Fig. 6b). The minor increase of sialidase activity in the sera of *nanA*$^{-}$*nanB*$^{-}$ -infected mice was likely due to release of host sialidases. Consistent with elevated serum sialidase activity, WT *S. pneumoniae* infections reduced spleen cell binding to Siglec 10Fc. In contrast, infection by *nanA*$^{-}$*nanB*$^{-}$ mutant had no effect on Siglec 10Fc binding (Fig. 6c). Therefore, bacterial sialidases were responsible for reduction of Siglec 10 binding in the spleen cells.

In WT mice, the WT *S. pneumonia*e was more virulent than the *nanA*$^{-}$*nanB*$^{-}$ mutant based on the onset of mortality (Fig. 6d). As expected, the *S. pneumonie* was more virulent in mice with mutation of either *Sigelcg* or *CD24*. However, deletion of sialidases had no effect on pathogenesis of the bacteria in the mutant mice. Consistent with mortality in WT mice, WT *S. pneumoniae* induced higher levels of IL-6 and TNFα than the *nanA*$^{-}$*nanB*$^{-}$ mutant 36 hours after infection, although the difference at 24 hours after infection was insignificant (Fig. 6e). In contrast, *nanA*$^{-}$*nanB*$^{-}$ mutant induced at least as much IL-6 and TNFα as the WT bacteria in *CD24*$^{-/-}$and *Siglecg*$^{-/-}$ mice 24 hours after infection. Due to loss of mutant mice at 36 hours, the cytokine levels in mutant mice were not measured after 24 hours to avoid sampling bias. As such, we were not able to assess correlations between mouse genotypes and the peak levels of inflammatory cytokines at the same time points. Analysis of CFU in the blood indicated that deletion of the sialidases did not reduce *S. pneumoniae* growth in vivo (Fig. 6f). Thus, the bacterial sialidases control virulence of *S. pneumonia*e by a growth-independent mechanism. Since the effect was abrogated in mice with mutation of either *CD24* or *Siglecg*, sialidases likely increase *S. pneumoniae* pathogenicity by targeting CD24-Siglec G interaction.

Discussion

Based on the observation that CD24-Siglec G/10 interaction selectively represses innate response to DAMPs [13], we proposed that that the interaction allowed the host to mount a more robust response to PAMPs than DAMPs [28]. Since infections often present both PAMPs and DAMPs simultaneously, it is of interest to determine the physiological function of CD24-Siglec G/10 interaction during infection. Here we use a polybacterial sepsis model to address this critical issue.

We chose the CLP model as it is the most commonly used sepsis model [19]. The disease starts with bacterial peritonitis. Multiple bacteria then enter into the blood to trigger systemic activation of the inflammatory response, septic shock, multiorgan dysfunction and death. Using mice with targeted mutation of either *Siglecg* or *CD24*, we showed that deletion of either gene exacerbates production of inflammatory cytokines and acute organ failure. Transgenic expression of CD24 predominantly on CD11c-expressing cells appears sufficient to repress the exacerbated virulence in the $CD24^{-/-}$ mice. However, a function of CD24 on other cell types, including $CD11c^{+}$ macrophages, cannot be ruled out. Furthermore, CD24 expressed on DC may interact with *Siglecg* in other cell types [15] to suppress production of inflammatory cytokines. Regardless of the cellular basis of CD24 function, our data demonstrated that CD24-Siglec 10/G interaction is a key regulator for polybacterial sepsis.

We presented evidence that CD24-Siglec G/10 interaction is disrupted by sialidases of multiple bacterial origins and can be restored by resialylation of CD24 with either α2–3 or α2–6 sialyltransferases. Thus, CD24 interacts with Siglec G/10 through sialoside-dependent pattern recognition. Sialidase is expressed in both gram positive and gram negative bacteria (Table S1). Since sialidase activity is increased in human sepsis patients [29], it is of interest to determine whether the sialidases are involved in pathogenesis. Since desialylation of DC increased their response to HMGB1 in WT but in not $CD24^{-/-}$ or $Siglecg^{-/-}$ mice, it is likely that its impact on host response to DAMPs is achieved by disruption of the CD24-Siglec G interaction. Importantly, a combination of two sialidase inhibitors conveyed a significant therapeutic effect. Again, the function of the inhibitors depends on CD24-Siglec G interaction as mutations of either gene abrogated its therapeutic effect.

Although the serum sialidase activity in CLP mice can be of host and/or microbial origin, our data suggest that bacterial sialidases are likely the major source of the activity. First, we documented transcripts of multiple bacterial sialidases in the peritoneal exudates of the CLP mice. Second, the elevation of sialidase activity was observed in CLP mice, but not in the sham surgery or LPS-treated mice, thus ruling out the possibility that inflammation alone was sufficient to cause elevation of sialidase. However, we have not ruled out the possibility that endogenous sialidase is released in the context of polybacterial sepsis. Nevertheless, it should be point out that as long as the sialidase-mediated disruption of CD24-Siglec G/10 interaction occurred selectively in the context of infection, whether the sialidase is of bacterial origin makes little difference to both pathogenesis and treatment of sepsis using sialidase inhibitors that do not discriminate bacterial and mammalian sialidase, as we have used herein.

On the other hand, genetic analyses using bacterial mutants demonstrated a critical role for sialidases in sepsis caused by a specific *S peumoniase.* Although the sialidase activity is still detectable in the serum of mice infected by the sialidase mutant , the levels are functionally insignificant as the *nanA*$^{-}$*nanB*$^{-}$ bacteria had no effect of Siglec 10-Fc binding to spleen cells. More importantly, despite equal blood bacterial burden, the *nanA*$^{-}$*nanB*$^{-}$ mutant exhibits reduced virulence in WT mice. The sialidases likely target CD24-Siglec G interaction as the *nanA*$^{-}$*nanB*$^{-}$ mutant is as virulent as the WT bacteria in the *CD24*$^{-/-}$ and *Siglecg*$^{-/-}$ mice. The essential requirement for the *CD24* and *Siglecg* genes in sialidase-mediated virulence, coupled with lack of effect of the sialidase on bacterial load in the blood, demonstrated that the *S. pneumoniae* sialidases are not required for optimal systemic infection. Grewal et al. reported that *Streptococcus pneumoniae* NanA may protect mice against intravascular coagulation during sepsis [30]. The different phenotype of the double mutant reported herein suggests that NanB may have a different function.

While our assay of blood bacterial burden under normoxia leaves open the possibility that genetic manipulation of mice can affect the growth of anaerobes, which are abundant in the CLP model, the facts that these manipulations similarly affect virulence in antibiotics-treated CLP mice and in mice infected with *S.pneumoniae* (which can be measured accurately by our assay) demonstrate a growth-independent role of CD24-Siglec G interaction. Nevertheless, it is possible that under different circumstances, the impacts of sialidases on bacterial growth and inflammation may work together to affect bacterial virulence. A more recent study demonstrated a role of desialyation in TLR4 signaling [31]. Since mutations of neither*Tlr4* [32] (and our unpublished observations) nor *Myd88* [33] attenuated mortality of CLP, it is unlikely that a direct impact of sialyation on TLR-4 signaling explains our data.

It is of note that many pathogens, including viruses and bacteria have their own sialidases, which are also known as neuraminidases [34, 35]. In both viruses and bacteria, the sialidases have been shown as virulence factors [24, 36-38], Until now, virulence and the sialidase function were largely viewed from the prism of pathogen growth. Our data presented herein have demonstrated that sialidase can regulate virulence by a bacterial growth-independent mechanism, i.e. by disruption of pattern recognition that selectively represses host response to tissue injuries [13, 28]. Since tissue injury is common during infections, it is likely that the sialidases from other pathogens also affect virulence by targeting CD24-Siglec 10 interaction.

The current paradigm on the cause of inflammation emphasizes interaction between PAMPs, DAMPs and their receptors [7, 39, 40]. Our data presented herein demonstrated that in addition to providing PAMPs and DAMPs, microbes may exacerbate innate immunity by disrupting sialic acid-based pattern recognition. This would further strengthen the discrimination between septic and aseptic insults [28]. Therefore, an effective strategy to control excessive inflammation must target both production of and regulation of host response to DAMPs and PAMPs. Since sepsis involves multiple inflammatory cytokines and other pathological changes [41], immunotherapies targeting individual cytokines have limited effect [42]. For that reason, it is always difficult to link a specific cytokine to mortality in this model. Our strategy to preserve the integrity of CD24-Siglec G/10 interaction by sialidase inhibitors

may have an advantage as multiple cytokines and complement are regulated by the interaction. Nevertheless, additional improvement of the sialidase inhibitors are likely needed before clinical development can be attempted.

Experimental procedure

Reagents

Recombinant protein consisting of human IgG Fc and extracellular domains of Siglec 10 and biotinylated anti-human Siglec 10 antibody were purchased from R&D Systems. Horseradish perioxidase conjugated anti-mouse IgG was purchased from Santa Cruz Biotechnology. Lipopolysaccharide (LPS, from *E. coli* 055:B5), α2-3 sialidase (N7271, from *S. pneumoniae*), and α2–3/6 sialidase (N5521, from *C. perfringens*) were purchased from Sigma (St Louis, MO). Sialidase (11082340, from *V. cholerae*) was purchased from Roche. Pierce Avidin Agarose beads were purchased from Thermo Scientific (Rockford, IL). Anti-mouse CD24-PE, anti-mouse CD11c-APC, and PE-streptavidin were purchased from eBioscience. Anti-human CD24 (Cat: 555426) were purchased from BD Pharmingen™. Amplex® Red Neuraminidase Assay Kit (A22178) was purchased from Molecular Probes. Neu5Acα2–3Lac [43] and Neu5Acα2–6Lac [44] were synthesized as described previously. Neu5Ac2en and Neu5Gc2en were synthesized from Neu5Ac and Neu5Gc[45], respectively, as described [46].

Experimental animals

CD24$^{-/-}$ and *Siglecg*$^{-/-}$ C57BL/6 mice have been described [15, 47]. Transgenic mice expressing CD24 under the control of CD11c promoter, CD24^{CD11ctg}, were produced using a previously described CD11c transgenic construct [22, 23]. Mice with DC-exclusive CD24 expression were produced by crossing the *CD24*^{CD11ctg} transgene into the *CD24*$^{-/-}$ background. All procedures involving mice have been approved by the University of Michigan Animal Care and Use Committee.

CLP

The procedure for lethal CLP was performed as described [19]. Briefly, mice were anesthetized with isoflurane anesthesia. Through a midline incision, the cecum was exteriorized and tightly ligated 1 cm from its base with 3-O silk. The cecum was then punctured through-and-through once with a 21 gauge needle. A small amount of stool was expelled from the puncture before the cecum was replaced into the peritoneal cavity and the abdominal incision closed. 100 μl sterile saline or sialidase inhibitors was administered by i.p. injection immediately after CLP.

In a less severe model, a 23G3/4 needle was used for puncture and unless specified otherwise, antibiotics was included. The antibiotics, imipenem (Merck, West Point, PA), was injected subcutaneously at 25 mg/kg, once every 12 hour, starting at 2 hours after surgery.

All CLP were performed blinded to the identities of the treatment groups and/or genotypes of the mice. Mortality was assessed twice a day for at least 14 days.

Generation of *nanA⁻nanB⁻ S. pneumoniae*

The *S. pneumoniae* strain D39 encodes two sialidases NanA and NanB. The gene encoding NanA is in a single gene transcriptional unit [48]. Therefore an insertion deletion mutation was constructed in the D39 Sm^r background as previously described and confirmed by PCR [49]. The gene encoding NanB is located in an operon therefore an unmarked non-polar mutation was constructed and confirmed by PCR and sequencing [50]. The absence of sialidase activity in the double mutant was confirmed by fluorescent activity assay performed in triplicate on three independent occasions.

Streptococcus pneumoniae infection

WT, *CD24*$^{-/-}$, *Siglecg*$^{-/-}$mice were infected with 10^4 CFU of either WT or *nanA⁻nanB⁻* mutant. Blood samples were obtained at given time points to determine the bacterial growth and inflammatory cytokines. Mice were observed every 12 hours for mortality.

Treatment of DC with sialidase

Bone marrow derived DC were generated as described. The DC cells were treated with sialidase (0.5 Unit/ml) or vehicle control for 1 hour at 37°C. After washing away the sialidase with PBS, the DC were stimulated with HMGB1 or HSP70 for 6 hours. Cytokines in the supernatants were measured by cytokine bead array.

Flow cytometric analysis for Siglec10 and Siglec G ligands

Spleen cells from normal WT or CLP treated WT mice were washed in buffer A (150 mM NaCl, 3 mM $MnCl_2$, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 2% BSA Tris-HCl, pH 7.6), and incubated for 1 hour on ice with 1 μg of Siglec10 Fc. The cells were washed and then incubated with biotinylated-anti-Siglec 10 antibody (0.05 μg/ml) for another hour on ice. The bound receptor was detected with PE conjugated streptavidin and analyzed on a BD LSII.

To prepare Siglec G-Fc or its mutant, the cDNA encoding extracellular portion of murine Siglec G (Met 19–Ser 528) was cloned into expression vector pFUSE-hIgG1-Fc2 (Invivogen). The double mutations (117Y>A; 120R>A) in the sialic acid-binding site of Siglec G were introduced by PCR-based site-directed mutagenesis. All constructs were verified by DNA sequencing. The expression vector was transfected into 293 cells using lipofectamine 2000 (Invitrogen). The cells were cultured in IgG-depeleted medium and the culture supernatants were collected for purification of the Siglec G Fc fusion protein. To detect Siglec G-Fc ligand, *Siglecg*$^{-/-}$ spleen cells were incubated with Siglec G-Fc (5 μg/ml), the bound fusion protein was detected by polyclonal mouse anti-Siglec G antisera [13] (1:100) followed by PE-conjugated goat anti-mouse IgG.

Measurement of inflammatory cytokines

Blood was obtained at indicated time points. Cytokines in the serum were determined using mouse cytokine bead array designed for inflammatory cytokines (Cat. No. 552364, BD Biosciences). The levels of C5a and MIF was measured by sandwich ELISA using kits from either BD Bioscience (C5a) or OncoImmune, Inc. (MIF), according the manufacturers' instruction.

Microplate binding assay

Ninety six-well plates were coated with either untreated, desialyated or resialylated CD24 in 50 mM carbonate/bicarbonate buffer, pH 9.5, overnight at 4°C. Wells were blocked with binding buffer (20 mM HEPES, 2% bovine serum albumin, 150 mM NaCl, 3 mM $MnCl_2$, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.6) for 1 hour. Siglec 10 Fc (1μg/ml) were added to the plate and incubated for 2 hours. Between incubations (all at 37°C), the plates were washed five times with the binding buffer. Biotinylated-anti-Siglec 10 antibody (0.05 μg/ml) was used to detect bound Siglec 10Fc. The plate-associated biotinylated proteins were detected by horseradish perioxidase (HRP)-conjugated streptavidin (1:1,000) for 1 hour and developed with 100 μl/well p-nitrophenyl phosphate liquid substrate system. Absorbance at 450 nm was recorded.

Immunoprecipitation and immunoblotting

Biotin conjugated human CD24Fc was digested with various kinds of sialidase for 16 hours at 37°C and then incubated with 1 μg/ml Siglec 10 Fc in buffer A. Siglec 10 bound to the CD24 was Immunoprecipitated with streptavidin-beads. Immunoprecipitates were washed 4 times with buffer A and re-suspended in SDS sample buffer for Western blot analysis.

Sialidase-desialylation and sialyltransferase-resialyation of CD24 were carried out as described in details in supplemental information.

Serum neuraminidase assay

The neuraminidase activity in serum was measured according to the procedure provided in Amplex® Red Neuraminidase Assay Kit (Cat. No. A22178).

Statistical analysis

The non-parametric Mann-Whitney T tests were used for pair-wise comparisons. The differences in survival rates were analyzed by Kaplan-Meier plot and the statistical significance was determined using a log-rank test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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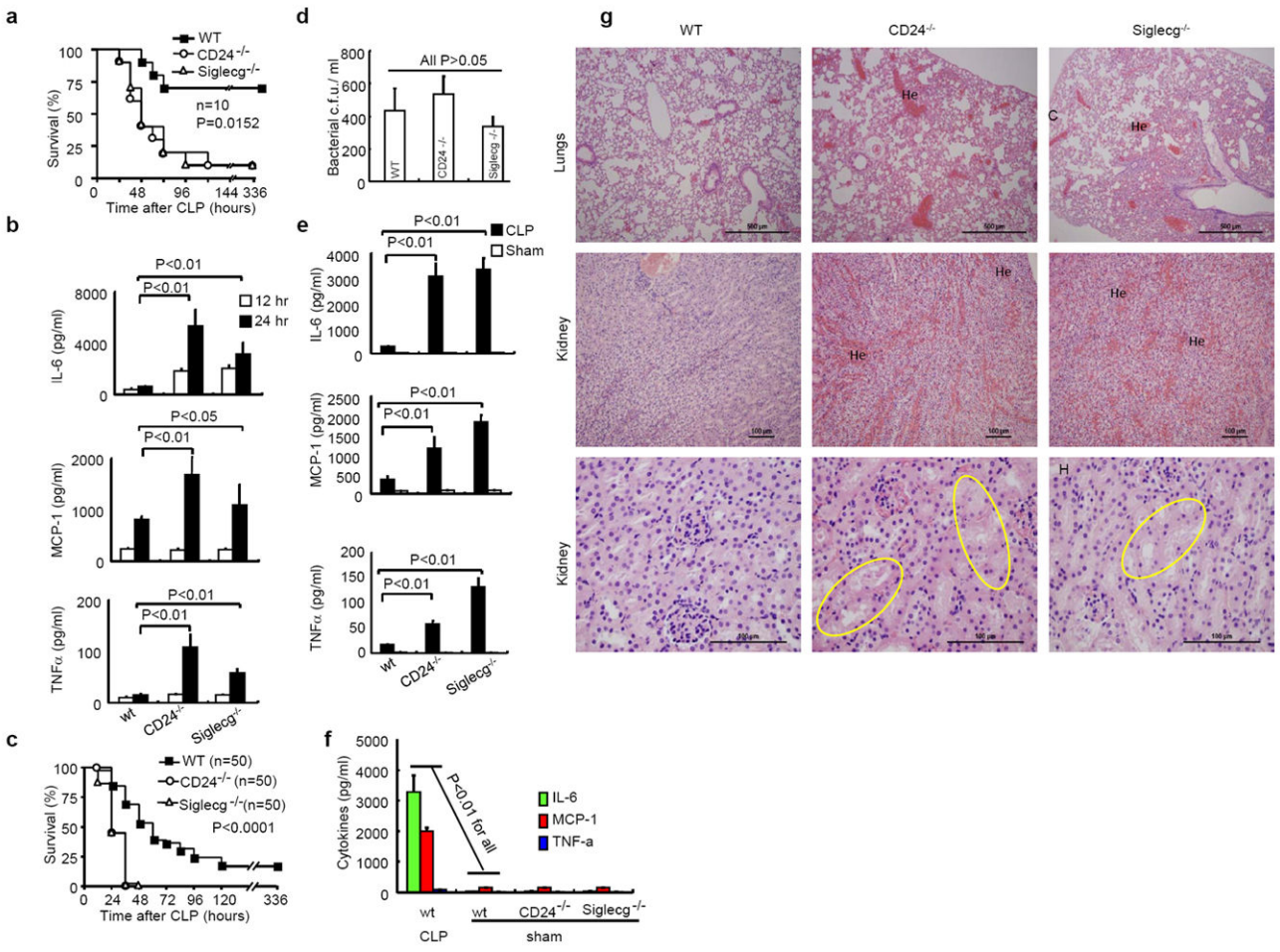

Fig. 1.

CD24 and Siglec G protect mice against inflammation and mortality associated with polybacterial sepsis. a. Targeted mutations of *CD24* or *Siglecg* genes increased mortality. Age-matched male mice received antibiotics and CLP using 23G3/4 needles. The mice were observed twice daily for 14 days. Data shown are Kaplan Meier analysis, with statistical significance determined by log rank test. b. Targeted mutation of either CD24 or Siglecg gene increased the production of inflammatory cytokines IL-6 and TNFα. Serum samples harvested at 12 or 24 hours after CLP were measured by cytokine beads array. Data are means+/-S.D. (n=5). c-g. Targeted mutation of either the *Siglecg* or the *CD24* gene exacerbates sepsis without increasing bacterial colony forming units (CFU) in the blood. The 21G needles were used and the CLP mice received no antibiotics. c. Survival of WT, *Cd24*$^{-/-}$, *Siglecg*$^{-/-}$ mice. The X-axis shows hours after CLP, while the Y-axis shows % of live mice. Data shown are summary of five experiments, each involving 10 mice per group. d. Bacterial burdens in the blood samples (CFU/ml) harvested at 12 hours after CLP (n=8). e. Elevation of inflammatory cytokines in mice with targeted mutation of either *CD24* or *Siglecg* at 12 hours after CLP (n=8). f. Inflammatory cytokines in the WT mice 24 hours after CLP. Data from mutant CLP mice were not collected due to mortality. g. *CD24*$^{-/-}$ and *Siglecg*$^{-/-}$ mice exhibit acute organ failures after CLP. Note increased alveolar and interstitial hemorrhage in lung (marked as He in top panel), massive hemorrhage and venous

congestion (marked as He in renal medulla and collecting tubules (middle panels), and focal tubular necrosis with vacuolar degeneration and nuclear pyknosis and karyolysis in kidney (marked by yellow circles), at 12 hours after CLP. All data presented have been validated by 2-5 independent experiments.

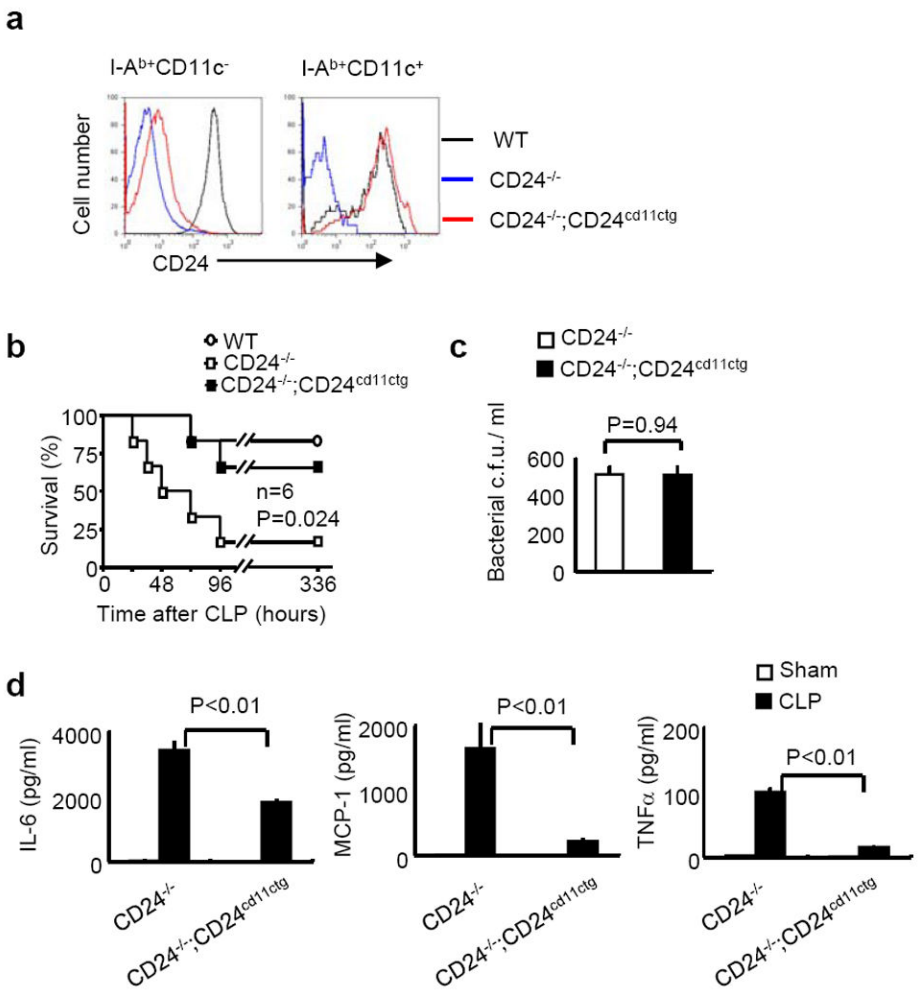

Fig. 2.

Expression of CD24 predominantly on dendritic cells (DC) conveys protection against sepsis. a. CD24$^{-/-}$ mice that expressed CD24 under the control of CD11c promoter, *CD24$^{-/-}$;CD24^{Cd11ctg}*. Data shown are FACS profiles depicting pattern of CD24 expression in the H-2I-A^{b+}CD11c^{-} and H-2I-A^{b+}CD11c^{+} splenocytes of WT, *CD24$^{-/-}$* and *CD24$^{-/-}$;CD24^{Cd11ctg}* mice. b. Expression of CD24 on DC increased mouse survival after CLP. A 23G3/4 needle was used for puncture and no antibiotics were used. *CD24$^{-/-}$;CD24^{cd11ctg}* mice and their CD24$^{-/-}$ littermates were treated by CLP and monitored for their survival. c. Transgenic expression of CD24 had no effect on blood bacterial burden at 24 hours after CLP (n=5). d. CD24 expression on DC suppressed production of inflammatory cytokines at 24 hours (n=5). The protection of DC-predominant CD24 against lethality and cytokine production has been observed in 4 independent experiments. CFU data are representative of those from two independent experiments.

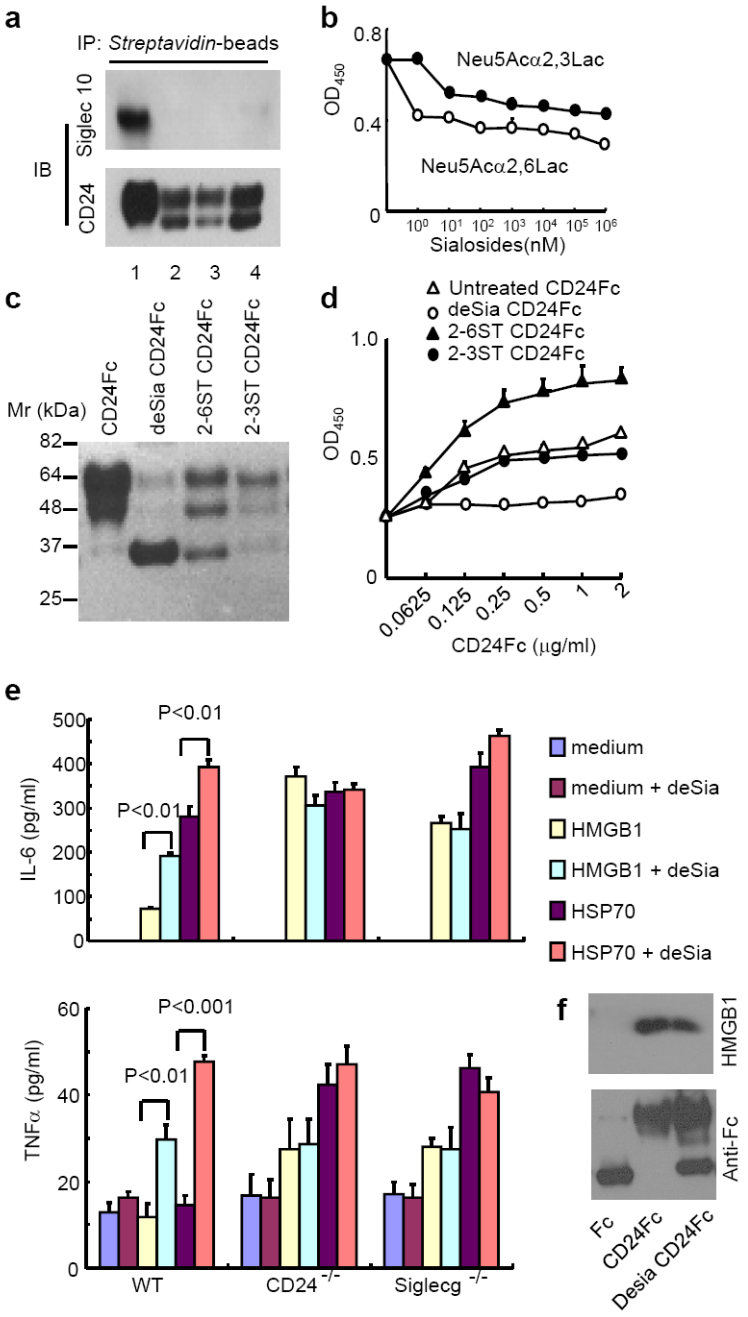

Fig. 3.

CD24-Siglec 10 interaction depends on sialyation of CD24. a. Biotinylated CD24Fc were pretreated with either control buffer (lane 1) or sialidase from *Streptococcus pneumoniae* (lane 2, specific for cleaving α2–3-linked sialic acids), *Clostridium perfringens* (lane 3, active for α2–6- or α2–3-linked sialosides), or *Vibrio cholerae* (lane 4, active for α2–3-, α2–6- or α2–8-linked sialosides) overnight at 37°C. The Siglec 10Fc fusion protein was incubated with the digested CD24Fc, and the complex was pulled down with streptavidin beads. The amounts of bead-bound Siglec 10Fc and CD24Fc were determined by Western blot with antibodies specific for either Siglec 10 or CD24. b. Efficient inhibition of CD24-

Siglec 10 interaction by sialosides. Siglec 10Fc were preincubated with given concentration of either Neu5Acα2–3Lac or Neu5Acα2–6Lac and then added to plate-bound CD24Fc. The CD24-bound Siglec 10Fc were measured by biotinylated anti-Siglec 10 followed by HRP-labeled streptavidin. c. Desialylation and resialylationof CD24Fc altered its electrophoresis mobility. d. Both α2–3- and α2–6-resialylations of CD24 restore Siglec 10Fc binding. e. Sialidase treatment of WT DC increase their response to HMGB1 and HSP70. Bone marrow-derived DC from WT, *CD24*$^{-/-}$ and *Siglecg*$^{-/-}$ mice were treated with sialidase prior to stimulation by either HMGB1 (1μg/ml) or HSP70 (7 nM). Cytokines in the supernatants were measured by cytokine beads array. f. Desialyation of CD24 barely reduced CD24Fc binding to HMGB1. Control IgG1Fc, untreated and desialyated CD24 were co-incubated with HMGB1 (1μg/ml). Protein A beads were used to pull down Fc. The amounts of HMGB1 associated with CD24Fc were determined by immunoblot with anti-HMGB1 mAb. The data shown are representative of 2-5 independent experiments.

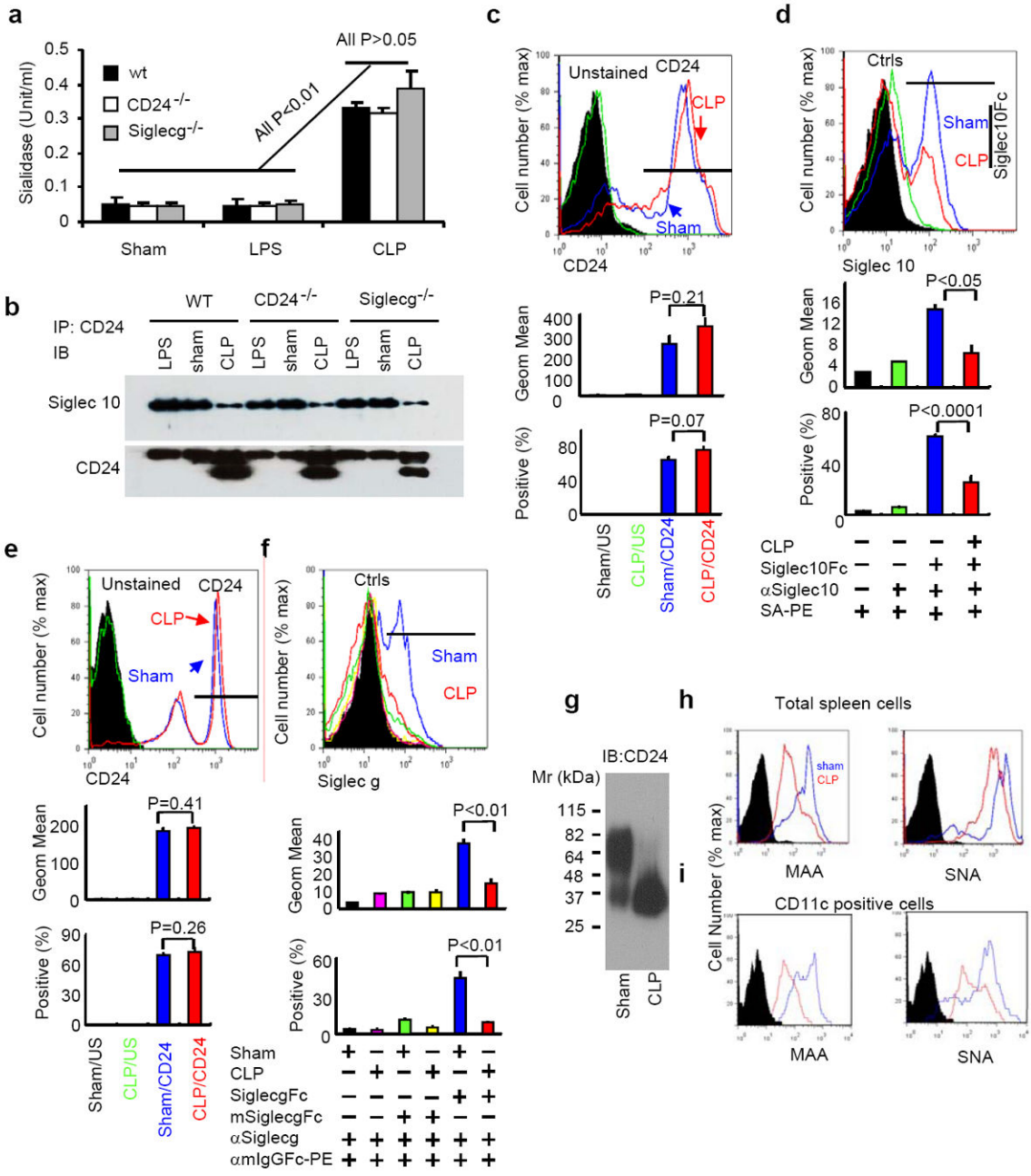

Fig. 4.

Increased circulating sialidase activity and reduction of Siglec 10 binding of CD24 in CLP mice. a. Sialidase activity in the sera of sham-, 100 μg/mouse LPS- or CLP-treated mice. Sera were collected at 12 hours after treatment (n=5). b. Pretreatment of biotinylated CD24Fc with sera from CLP mice reduced its binding to Siglec 10Fc. Data shown are co-IP with streptavidin-conjugated beads. The top panel shows the amounts of Siglec 10Fc in the precipitates as determined by Western blot. The molecular weight shift of CD24Fc is demonstrated by Western blot using HRP-labeled streptavidin in the bottom panel. c. CLP does not affect CD24 expression in spleen cells. US, unstained; SA-PE, phycoerythorin-

conjugated streptavidin. d. CLP significantly reduced spleen cell binding to Siglec 10Fc. Histograms shown on top panels are FACS profiles depicting distribution of CD24 in sham-surgery (blue line) or CLP (red line) spleen cells. The bar graphs in the bottom panels present means+/-S.D. of mean fluorescence intensities (n=3). The gates used to determine % positive cells were labeled in the upper panels. e. f. CLP reduce spleen cell binding to WT and mutant Siglec G-Fc (f) without affecting the total CD24 levels (e). The bar graphs in the bottom panels present means+/-S.D. of geo-mean fluorescence intensities or % positive cells (n=3). g. CLP alters the molecular weight distribution of CD24 in the spleen cell lysates, as determined by Western blot. h &i. CLP reduces both α2–3- and α2–6-sialyation of spleen cells (h) and $CD11c^+$ cells (i). MAA: Fluorescein-*Maackia amurensis* Lectin I, recognizing α2–3-linked terminal sialic acid. SNA: Fluorescein-*Sambucus nigra* (Elderberry) Bark Lectin (SNA), recognizing α2–6-linked terminal sialic acid. All data are representative of 2–3 independent experiments.

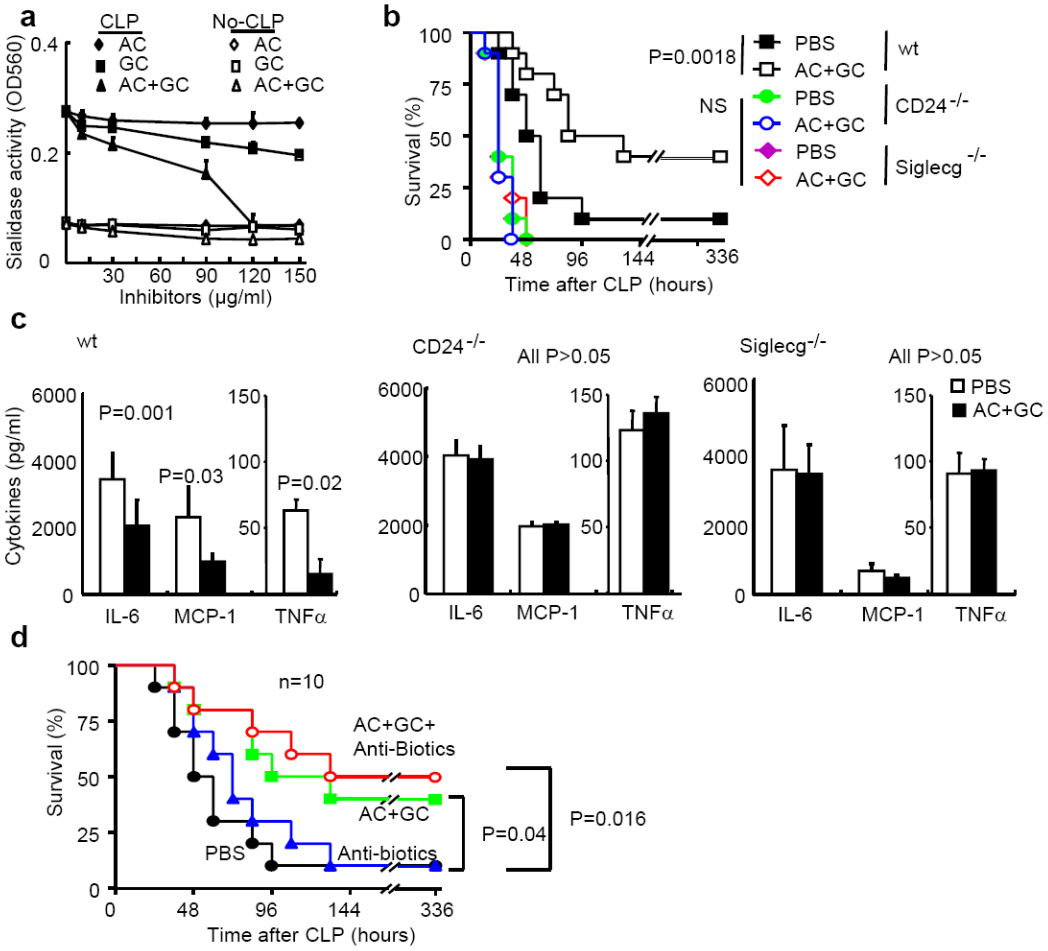

Fig. 5.

Sialidase inhibitors protect mice against sepsis. a. A mixture of two sialidase inhibitors blocks serum sialidase activity. Sera from CLP mice were mixed with given doses of inhibitors, Neu5Ac2en (AC), Neu5Gc2en (GC), or both (AC+GC) prior to the assay. The sialidase activity was measured using the Amplex Red Neuraminidase assay kit. Data shown are means+/-S.D. of triplicates. b. Sialidase inhibitors prolong survival of WT but not CD24$^{-/-}$ and Siglecg$^{-/-}$ mice after CLP (n=10). The mice received a mixture of AC and GC (100 μg/mouse/injection) immediately prior to CLP and every 12 hours thereafter. c and d. Sialidase inhibitors had no effect on the serum bacterial CFU at 12 hours (c, left panel) or 24 hours (c, right panel) after CLP. Bacterial burden in mutant mice 24 hours after CLP were not assayed as mouse deaths biases sampling. d. Sialidase inhibitors reduce the levels of multiple inflammatory cytokines. Sera were collected at 24 hours after CLP to measure cytokines. Data shown are means+/-S.D. (n=8). e. Sialidase inhibitor protects CLP mice in combination with antibiotics. Data shown are representative of 2–5 independent experiments.

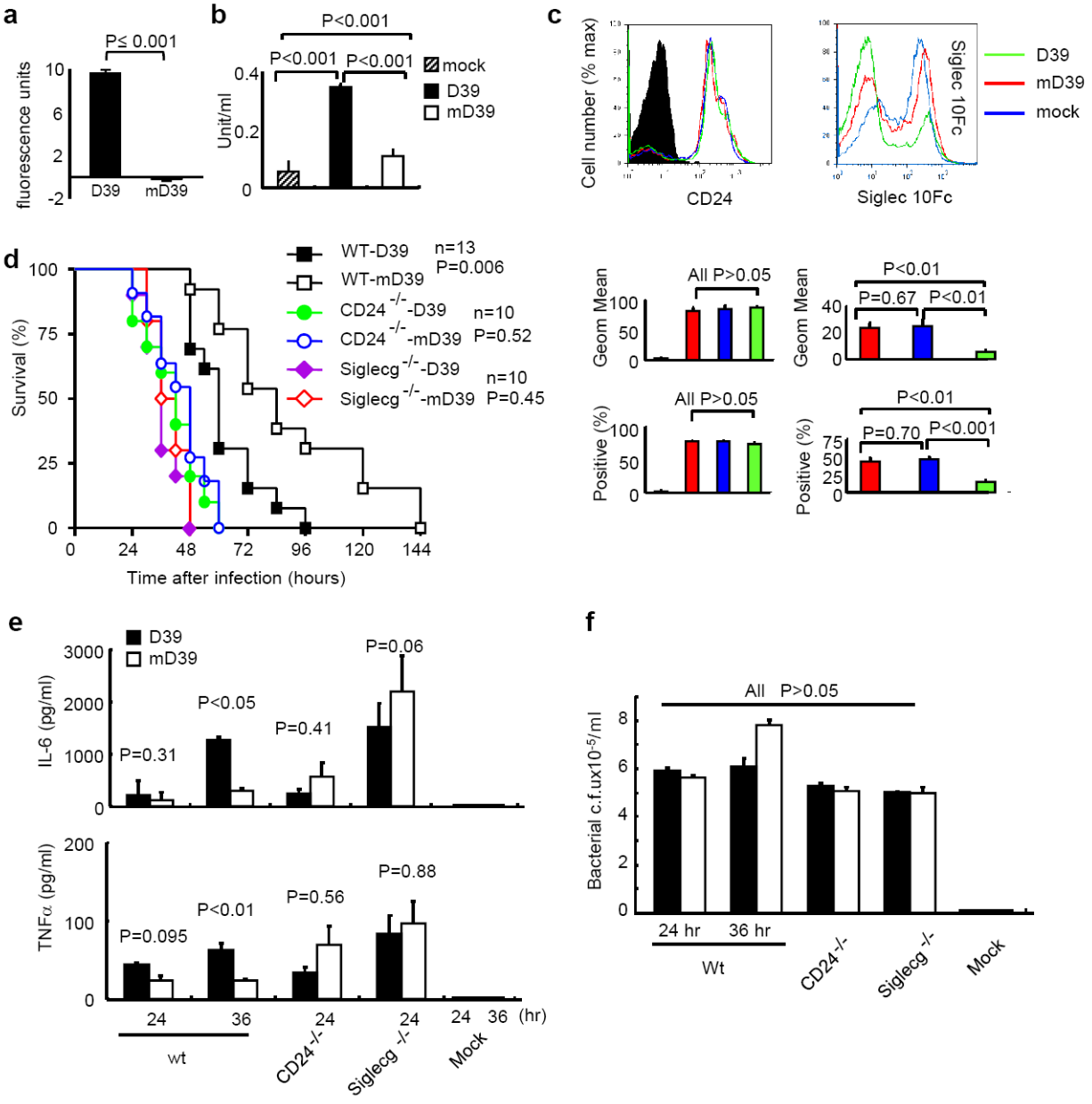

Fig. 6.

S. pneumoniae sialidases exacerbate sepsis by CD24-and Sigelec G-dependent mechanism. a. Characterization of the sialidase activity of WT (D39) and *nanA*$^{-}$*nanB*$^{-}$ mutant (mD39) strains. b. Serum sialidase activity of mice at 24 hours after intraperitoneal infections with 10^4 CFU of either D39 or mD39. The sialidase activity in a and b were measured using Amplex® Red Neuraminidase Assay Kit. c. Bacterial sialidase reduces Siglec 10Fc binding to spleen cells. Representative FACS profiles are shown in the top panels, while the summary data were shown at the lower panel (n=3). d. Bacterial sialidases exacerbate sepsis in WT but not mutant mice. Data shown were Kaplan Meier survival analysis of accumulating data from two independent experiments (n=10), the log-rank tests were used to calculate the P value. e. Bacterial sialidases increased production of inflammatory cytokines

in WT but not mutant mice. f. Deletion of gene encoding both NanA and NanB sialidases does not reduce blood bacterial burden. Data shown represents 2–3 independent experiments. N=5 unless otherwise specified.