Siglec-5 and Siglec-14 are polymorphic paired receptors that modulate neutrophil and amnion signaling responses to group B *Streptococcus*

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Group B *Streptococcus* (GBS) causes invasive infections in human newborns. We recently showed that the GBS β-protein attenuates innate immune responses by binding to sialic acid–binding immunoglobulin-like lectin 5 (Siglec-5), an inhibitory receptor on phagocytes. Interestingly, neutrophils and monocytes also express Siglec-14, which has a ligand-binding domain almost identical to Siglec-5 but signals via an activating motif, raising the possibility that these are paired Siglec receptors that balance immune responses to pathogens. Here we show that β-protein–expressing GBS binds to both Siglec-5 and Siglec-14 on neutrophils and that the latter engagement counteracts pathogen-induced host immune suppression by activating p38 mitogen-activated protein kinase (MAPK) and AKT signaling pathways. Siglec-14 is absent from some humans because of a *SIGLEC14*-null polymorphism, and homozygous *SIGLEC14*-null neutrophils are more susceptible to GBS immune subversion. Finally, we report an unexpected human-specific expression of Siglec-5 and Siglec-14 on amniotic epithelium, the site of initial contact of invading GBS with the fetus. GBS amnion immune activation was likewise influenced by the *SIGLEC14*-null polymorphism. We provide initial evidence that the polymorphism could influence the risk of prematurity among human fetuses of mothers colonized with GBS. This first functionally proven example of a paired receptor system in the Siglec family has multiple implications for regulation of host immunity.

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Abbreviations used: AM, amniotic epithelial membrane; GBS, group B *Streptococcus*; ITIM, immunoreceptor tyrosine-based inhibitory motif; MAPK, mitogen-activated protein kinase; MFI, mean fluorescence intensity; MOI, multiplicity of infection; NET, neutrophil extracellular trap; ROS, reactive oxygen species; Siglec, sialic acid–binding immunoglobulin-like lectin.

Microbial pathogens have evolved many ingenious ways to evade host innate immune responses and phagocytic clearance (Flannagan et al., 2009; Diacovich and Gorvel, 2010; Neish and Naumann, 2011). Because all microbial and host cell surfaces are decorated with carbohydrate structures, glycan–receptor interactions play crucial roles in microbial pattern recognition as well as in the regulatory signals that govern normal immune cell activities (Comstock and Kasper, 2006). Consequently, a key virulence strategy of many leading pathogens is to display sugars in a fashion that mimics or interferes with host glycan–based immune functions (Nizet and Esko, 2009).

Sialic acids are 9-carbon-backbone sugars found in abundance on terminal glycan structures of mammalian cell surfaces, including all human cells. Human leukocytes express various members of a rapidly evolving receptor family known as CD33-related sialic acid–binding immunoglobulin-like lectins (Siglecs); sialylated glycans bind to Siglecs and modulate their function. Because many CD33-related Siglecs have an immunoreceptor tyrosine-based inhibitory motif (ITIM) and/or ITIM-like motifs in their intracellular domain, engagement of sialic acid–containing glycans can mediate an inhibitory signal (Crocker et al., 2007). Consequently,

The Rockefeller University Press $30.00
J. Exp. Med. 2014 Vol. 211 No. 6 1231-1242
www.jem.org/cgi/doi/10.1084/jem.20131853

endogenous sialoglycans represent the first example of a widely distributed "self-associated molecular pattern" (SAMP) that can act to dampen leukocyte activation under homeostatic conditions (Varki, 2011).

Several important human pathogens including group B *Streptococcus* (GBS), *Haemophilus influenzae*, *Escherichia coli* K1, *Neisseria gonorrhoeae*, and *Trypanosoma cruzi* incorporate sialic acids into their terminal glycans, either scavenged directly from host or synthesized de novo (Nagamune et al., 2004; Crocker et al., 2007; Severi et al., 2007; Carlin et al., 2009b; Cao and Crocker, 2011). Such molecular mimicry of host sialylated glycans allows the bacterial pathogen to engage inhibitory Siglecs, dampen leukocyte activation, and attenuate immune clearance (Carlin et al., 2009b; Diacovich and Gorvel, 2010; Hajishengallis and Lambris, 2011; Varki and Gagneux, 2012). For example, capsular serotype III GBS engages Siglec-9 on human neutrophils to block the oxidative burst, neutrophil extracellular trap (NET) production, and bacterial killing, and the sialylated *Campylobacter jejuni* lipooligosaccharide core binds Siglec-7 on dendritic cells to modulate T cell polarization (Carlin et al., 2009b; Bax et al., 2011). In addition to sialic acid–dependent binding, certain GBS strains can also engage Siglec-5 through β-protein, which is anchored to and extends from the bacterial cell wall (Carlin et al., 2009a; Nordström et al., 2011). GBS β-protein engagement of ITIM-bearing Siglec-5 initiates inhibitory SHP2-dependent signals that interfere with macrophage activation and phagocytic killing (Carlin et al., 2009a).

The fitness costs of pathogen sialic acid mimicry or protein-based engagement of inhibitory Siglecs may drive evolutionary changes in the host sialic acid repertoire or Siglec-binding specificity (Varki, 2009; Varki and Gagneux, 2012; Wang et al., 2012b; Padler-Karavani et al., 2014). One such evolutionary adaptation may be the emergence of activating Siglecs that hypothetically could function to counteract pathogen immune evasion. In this regard, it is notable that all known primate *SIGLEC5* genes are undergoing partial gene conversions with the adjacent gene *SIGLEC14*. Siglec-14 is thus nearly identical to Siglec-5 in its ligand-binding domain, but associates with activating DAP12 adaptor protein bearing ITAM instead of the inhibitory ITIM on the cytosolic side (Angata et al., 2006). Interestingly, functional Siglec-14 is missing from certain human individuals because of a fusion between the *SIGLEC5* and *SIGLEC14* genes leading to a new gene (*SIGLEC5**; hereafter referred to as *SIGLEC14/5*), which is identical to *SIGLEC5* in the coding sequence but is expressed under control of the *SIGLEC14* promoter (Yamanaka et al., 2009). This leads to a polymorphism within the human population, where individuals either possess both *SIGLEC5* and *SIGLEC14* or lack one or both alleles of *SIGLEC14* as the result of its replacement by the *SIGLEC14/5* fusion gene.

Recently, we reported that the *SIGLEC14* gene polymorphism influenced the susceptibility of chronic obstructive pulmonary disease (COPD) patients to exacerbations within a Japanese cohort (Angata et al., 2013). However, whether this human gene polymorphism affects the host innate immune defense and bacterial pathogenesis is not known. Furthermore, it is uncertain whether Siglec-5 and Siglec-14 function independently or as paired receptors.

Currently known paired receptors are indeed activating and inhibitory membrane receptors with similar ligand binding domains (Arase and Lanier, 2004; Skokowa et al., 2005). Upon activation, they induce signaling pathways in opposite directions for a balanced immune response (Arase and Lanier, 2004). Although the concept of paired receptors was previously suggested for Siglec-5 and Siglec-14 as well as Siglec-11 and Siglec-16, it has never been functionally proven in native cells (Angata et al., 2006; Crocker et al., 2007; Cao and Crocker, 2011; Pillai et al., 2012).

In this work, we study how the presence or absence of Siglec-14 influences leukocyte responses to GBS that express the Siglec-5–binding β-protein. This study is conducted through controlled expression of the respective Siglec receptors in cultured macrophages in vitro and by ex vivo analysis of blood cells from humans harboring different genotypes at the *SIGLEC14* locus. Our findings demonstrate that Siglec-14 can indeed serve to counterbalance the suppressive effects of GBS β-protein–mediated engagement of Siglec-5 on leukocyte activation and reveal roles of mitogen-activated protein kinases (MAPKs) and AKT in differential downstream signaling. Although Siglecs are appreciated primarily as leukocyte receptors, we also uncover a surprising human-specific expression of Siglec-5 and Siglec-14 on the fetal amnion. The human *SIGLEC14/5* polymorphism likewise influences inflammatory responses of amniotic epithelium to GBS, a neonatal pathogen which gains access to the fetus through placental membranes. An initial analysis of the *SIGLEC14/5* polymorphism among premature newborns and their mothers suggests that loss of Siglec-14 may influence the incidence of premature delivery.

RESULTS

Expression of Siglec-14 on THP-1 monocytes increases responsiveness to LPS and GBS

It has been suggested but not proven that Siglec-5 and Siglec-14 are paired receptors that could mediate opposing effects on leukocyte responses to bacterial products or bacterial receptor engagement. Our prior finding demonstrates that type 1a GBS β-protein can engage Siglec-5 and deliver an inhibitory signal to leukocytes; however, its interaction with Siglec-14 remained unknown (Carlin et al., 2009a). In keeping with the nearly identical ligand-binding domains of the two Siglecs, we found that GBS β-protein bound equally strongly to Siglec-14 Fc-chimera and Siglec-5 Fc-chimera (not depicted). To conduct an initial in vitro analysis, we used THP-1 (human monocyte like) cells that express only low levels of endogenous Siglec-5 and lack Siglec-14 protein (not depicted). As described earlier, these cells were stably transfected with vectors expressing Siglec-5 (THP-1–Siglec-5), Siglec-14 (THP-1–Siglec-14), or empty vector control (THP-1–EV; Yamanaka et al., 2009). We observed an increased GBS binding

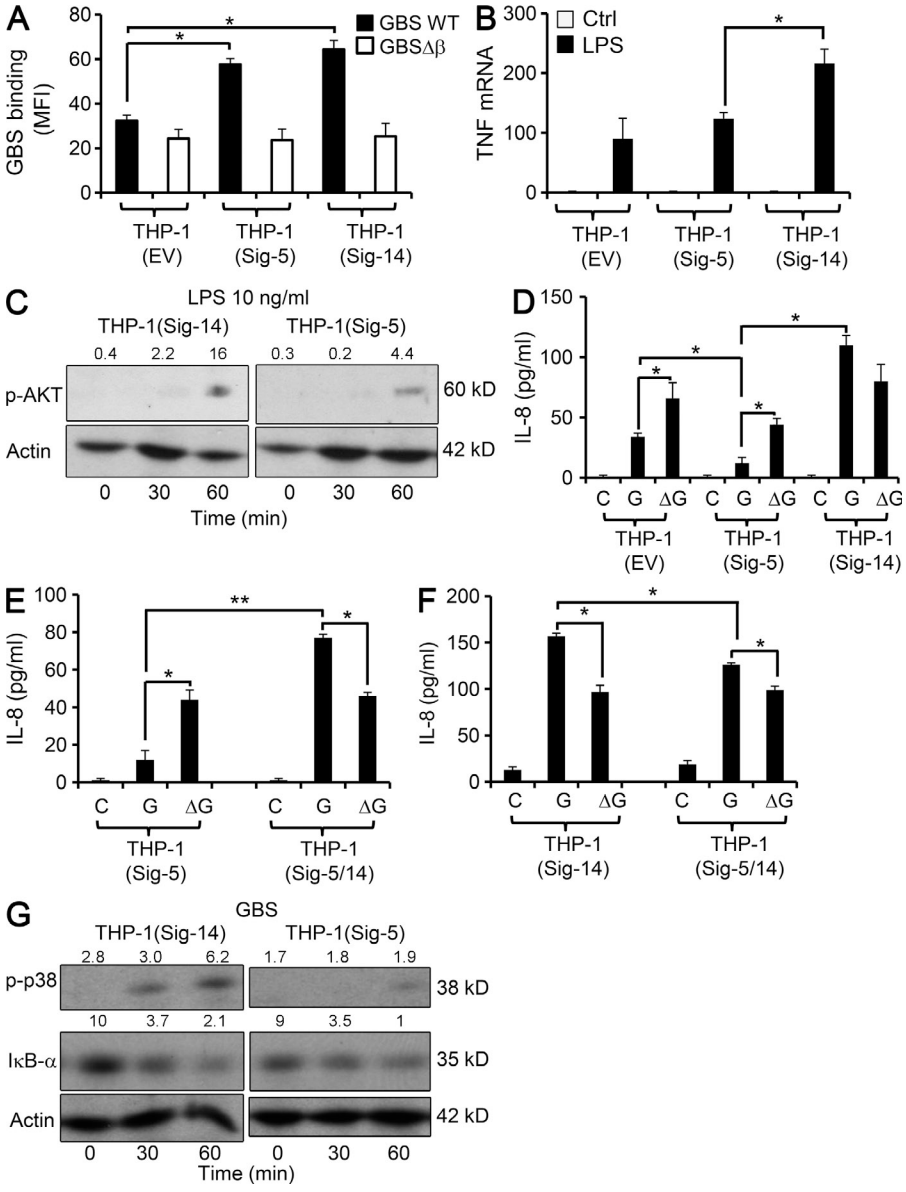


Figure 1. Expression of Siglec-14 on THP-1 monocytes increases responsiveness to LPS and GBS. (A) THP-1 cells expressing Siglec-5 (THP-1–Sig-5), Siglec-14 (THP-1–Sig-14), or empty vector (THP-1–EV) were infected with FITC-labeled GBS or with GBSΔβ at 4°C, and then bacterial binding to neutrophils at the 20-min time point was analyzed by FACS. The graph depicts MFI of GFP fluorescence on neutrophils. (B) The indicated THP-1 cell variants were stimulated with 10 ng/ml LPS or media control for 2 h, and TNF mRNA was measured by Q-RT-PCR and normalized to GAPDH mRNA. (C) The indicated THP-1 cell variants were stimulated with or without 10 ng/ml LPS for the indicated times, lysed, and analyzed for AKT phosphorylation by immunoblot. The p-AKT/actin (×10) densitometry value is shown on the immunoblot. (D–F) IL-8 protein was measured in the supernatant of uninfected THP-1 cell variants and those infected with GBS or GBSΔβ (MOI = 10) for 6 h. (G) The indicated THP-1 cell variants were infected or not with GBS, and cell lysates were prepared and analyzed for p38 phosphorylation and IκB-α degradation by immunoblotting. The p-p38/actin and IκB-α/actin (×10) densitometry value is shown on corresponding blots. All data are representative of two to three independent experiments. Results are means ± SD; *, $P < 0.05$, **, $P < 0.01$.

to THP-1–Siglec-5 and THP-1–Siglec-14 cells compared with THP-1–EV control cells (Fig. 1 A). Previous work has shown that LPS-triggered cell activation is altered by the surface expression of Siglec-5, -7, -9, -11, and -14 by unknown mechanisms (Lock et al., 2004; Wang and Neumann, 2010; Pillai et al., 2012). Consistent with prior findings (Yamanaka et al., 2009), LPS administration stimulated more TNF mRNA production from THP-1–Siglec-14 compared with THP-1–Siglec-5 and empty vector control (Fig. 1 B). Siglec-14 is associated with DAP12 (DNAX activation protein of 12 kD), which is known to signal through phosphoinositide-3-kinase (PI3K)/Akt pathways (Angata et al., 2006). Enhanced AKT activation, as indicated by immunoblot for AKT S473 phosphorylation, was observed in THP-1–Siglec-14 compared with THP-1–Siglec-5 cells (Fig. 1 C). To investigate the role of Siglec-5 and Siglec-14 in the innate immune response to GBS, THP-1 cell variants were infected with GBS at a multiplicity of infection (MOI) of 10; GBSΔβ infections served as negative control for β-protein–mediated effects. Interestingly, THP-1 cells expressing Siglec-14 (THP-1–Sig-14) exhibited increased IL-8 protein secretion when infected with WT versus mutant GBS ($P = 0.06$). In contrast, THP-1–Sig-5 cells produced less IL-8 protein when infected with WT versus mutant GBS (Fig. 1 D). Importantly, IL-8 production was significantly enhanced in THP-1–Sig-14 compared with THP-1–Sig-5 cells (Fig. 1 D). The enhanced IL-8 protein production by THP-1–EV cells infected with WT versus mutant GBS was likely caused by endogenous Siglec-5 expression. Moreover, THP-1–Siglec-5/14 (THP-1 cells overexpressing Siglec-5 and Siglec-14; Angata et al., 2013) produced elevated IL-8 compared with THP-1–Sig-5 but reduced IL-8 compared with THP-1–Sig-14 cells, in response

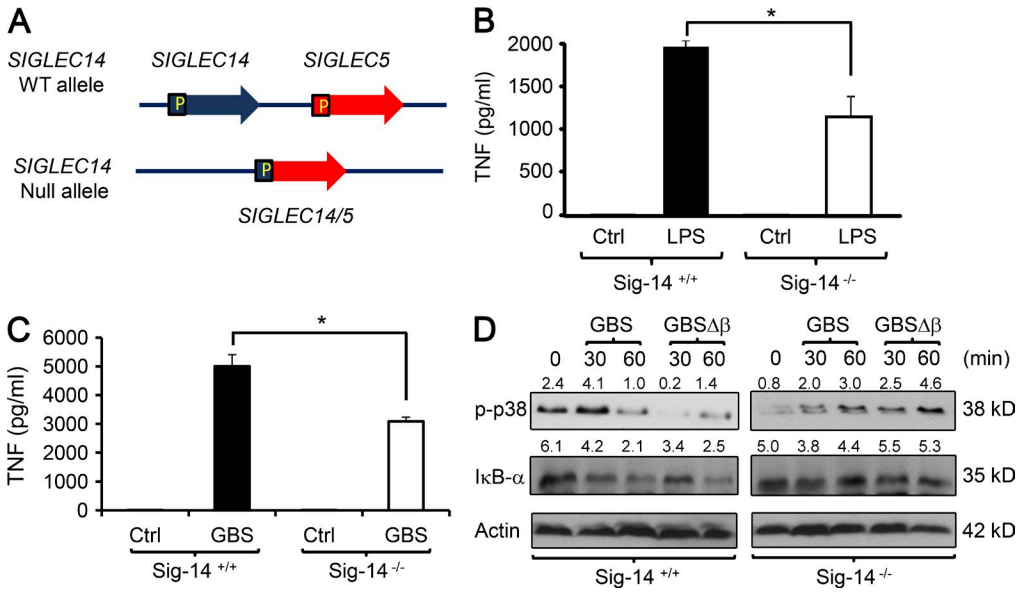


Figure 2. The Siglec-5/14 genotype influences primary human monocyte responses to LPS and GBS. (A) Pictorial representation of *SIGLEC-14/5* polymorphism in humans. Highly similar regions of *SIGLEC14* and *SIGLEC5* resulted in the generation of a *SIGLEC14/5* fusion gene, leading to deletion of *SIGLEC14* and expression of *SIGLEC5* under the *SIGLEC14* promoter. (B) Human blood monocytes of the indicated genotypes were left unstimulated (Ctrl) or stimulated with 10 ng/ml LPS, and TNF protein release was measured at 6 h by ELISA. (C) Human blood monocytes of the indicated genotypes were left uninfected (Ctrl) or infected with GBS at MOI = 10, and TNF protein release was measured at 6 h by ELISA. (D) Human monocytes of the indicated genotypes were left uninfected or infected with GBS or GBSΔβ for the indicated times and then lysed and analyzed for phosphorylation of p38 MAPK and IκB-α degradation by immunoblot. The p-p38/actin and IκB-α/actin (×10) densitometry values are shown on corresponding blots. Data in B–D are representative of two to four independent experiments with one to three different donors per genotype in each experiment. Results are means ± SD; *, $P < 0.05$.

to GBS but not GBSΔβ (Fig. 1, E and F). Thus, our results demonstrate an activatory role of Siglec-14 in GBS β-protein–mediated responses.

Infection with GBS also led to enhanced p38 MAPK phosphorylation in THP-1–Siglec-14 compared with THP-1–Siglec-5 cells (Fig. 1 G). In contrast, similar amounts of NF-κB activation were observed across the THP-1 cell variants after GBS challenge, as indicated by similar disappearance of the IκB-α protein (Fig. 1 G). Together, our results demonstrate that expression of Siglec-14 on THP-1 cells increases responsiveness to LPS and GBS.

SIGLEC14/5 gene polymorphism influences primary human monocyte responses to LPS and GBS

Based on the in vitro THP-1 experiments, we hypothesized that the human-specific *SIGLEC14/5* gene polymorphism could influence the responsiveness of primary human monocytes to infectious agents. The 5′ regions of *SIGLEC5* and *SIGLEC14* exhibit high similarity as the result of ongoing gene conversion, and in some individuals, the two are fused into a single gene that encodes for a functionally Siglec-5 gene product expressed under the *SIGLEC14* promoter (Fig. 2 A; Yamanaka et al., 2009). The homozygous state of this allele is hereafter referred to as Sig-$14^{-/-}$, whereas the homozygous state of the ancestral WT alleles carrying Siglec-5 and Siglec-14 is denoted as Sig-$14^{+/+}$. Genotype analysis of blood cells was performed and analyzed as described previously (Yamanaka et al., 2009), and cells from three to four donors with homozygous genotypes were used in this study; separate experiments were performed with each donor sample. Primary monocytes from Sig-$14^{+/+}$ individuals showed elevated TNF production compared with monocytes from Sig-$14^{-/-}$ individuals after challenge with LPS (Fig. 2 B) or GBS (Fig. 2 C). To investigate the role of Siglec-5 and Siglec-14 in primary human monocytes, Sig-$14^{+/+}$ and Sig-$14^{-/-}$ cells were infected with GBS at an MOI of 10; GBSΔβ infections served as negative control for β-protein–mediated effects. We observed increased p-p38 activation in Sig-$14^{+/+}$ cells with WT versus mutant GBS infection, likely because of activation of Siglec-14 by WT bacteria. Indeed, as predicted, p-p38 activation was reduced in Sig-$14^{-/-}$ cells with WT versus mutant GBS infection (Fig. 2 D). Moreover, unlike in THP-1 cells (Fig. 1 F), we also observed a moderate increase in NF-κB activation, as indicated by the disappearance of IκB-α in GBS-exposed Sig-$14^{+/+}$ compared with Sig-$14^{-/-}$ monocytes; GBSΔβ induced similar NF-κB activation in cells of both genotypes (Fig. 2 D). Collectively, results from THP-1 cells (Fig. 1) and from monocytes from healthy human donors without or with *SIGLEC14/5* polymorphism (Fig. 2) indicate that Siglec-14 promotes proinflammatory responses to LPS and GBS through activation of AKT and/or p38 MAPK pathways, thus providing an opposing force to Siglec-5, which acts via a cytosolic ITIM motif that recruits the tyrosine phosphatases SHP-1 and SHP-2 (Carlin et al., 2009a).

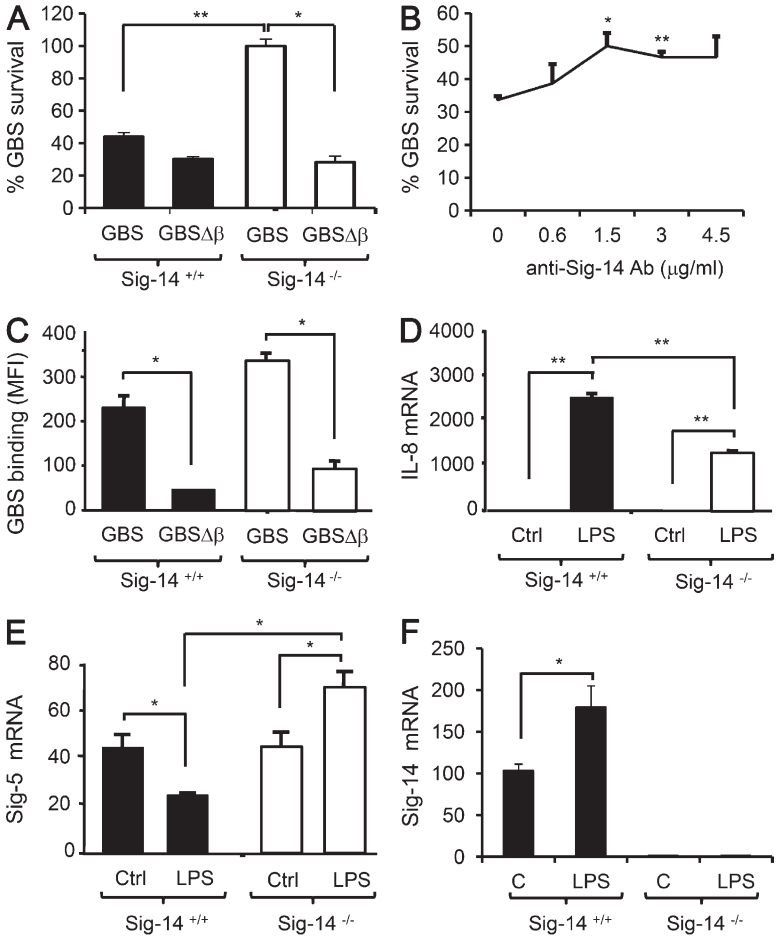


Figure 3. The Siglec-5/14 genotype influences human neutrophil responses to GBS. (A) Human neutrophils of the indicated genotypes were left uninfected or infected with GBS or GBSΔβ (MOI = 5), and bacterial survival at 40 min was assessed by CFU enumeration. (B) Sig-14$^{+/+}$ neutrophils were pretreated with the indicated concentrations of anti–Siglec-14 antibody (Ab) for 15 min, followed by GBS infection as in A; after 40 min, bacterial survival was assayed by CFU enumeration. Data are representative of three independent experiments with one donor in each experiment. (C) Human neutrophils of the indicated genotypes were infected with FITC-labeled GBS or with GBSΔβ at 4°C, and then bacterial binding to neutrophils at the 20-min time point was analyzed by FACS. The graph depicts MFI of GFP fluorescence on neutrophils. (D–F) Human neutrophils of the indicated genotypes were left unstimulated or stimulated with LPS, and IL-8 (D), Siglec-5 (E), and Siglec-14 (F) mRNA were analyzed by Q-RT-PCR at 2 h. Results were normalized to the amount of GAPDH mRNA. Data in this figure are representative of two to four independent experiments with one to three different donors per genotype in each experiment. Results are means ± SD; *, $P < 0.05$, **, $P < 0.01$.

SIGLEC14/5 gene polymorphism influences human neutrophil responses to GBS

Neutrophils are the critical first line phagocytic cells in innate immune defense against invasive bacterial pathogens. Sig-14$^{+/+}$ human neutrophils killed GBS more efficiently than those isolated from Sig-14$^{-/-}$ individuals (Fig. 3 A). The difference in killing of GBS and GBSΔβ in Sig-14$^{+/+}$ neutrophils was not statistically significant, probably because of the offsetting effect of endogenous Siglec-5. To confirm that the reduced GBS killing by Sig-14$^{-/-}$ neutrophils reflects the absence of Siglec-14 by itself, the Siglec-14 activity of Sig-14$^{+/+}$ neutrophils was disrupted using an antibody that specifically recognizes Siglec-14 but not Siglec-5 (Yamanaka et al., 2009). Anti–Siglec-14 antibody–treated neutrophils killed GBS less efficiently than neutrophils treated with an isotype control antibody in a dose-dependent manner (Fig. 3 B). These differences in cytokine release and killing do not reflect differential overall binding of GBS to the neutrophil surface, which was similar in Sig-14$^{+/+}$ and Sig-14$^{-/-}$ neutrophils and dependent on β-protein expression (Fig. 3 C). Upon LPS challenge, neutrophils from human donors with the Sig-14$^{+/+}$ genotype also produced significantly higher levels of IL-8 transcript than Sig-14$^{-/-}$ neutrophils (Fig. 3 D).

Given the nearly identical ligand-binding domains and opposing intracellular functions, Siglec-5 and Siglec-14 may thus indeed act as a paired receptor system (Crocker et al., 2007; Pillai et al., 2012). Balanced activities of other paired activating and inhibitory receptors are critical for effective immune responses (Kumar et al., 2006; Barclay and Hatherley, 2008). One mechanism underlying such a balance is the coordinated regulation of receptor expression; for example, the inhibitory receptor is down-regulated at the same time that the activating receptor is up-regulated (Skokowa et al., 2005; Kumar et al., 2006). Primary human neutrophils of the indicated genotypes were treated with LPS for 2 h, and receptor mRNA was quantified by Q-RT-PCR. In Sig-14$^{+/+}$ neutrophils, *SIGLEC5* mRNA was down-regulated, whereas *SIGLEC14* mRNA was up-regulated in response to LPS (Fig. 3, E and F). Similar results were obtained when neutrophils were stimulated with a TLR2 ligand, PamCys3K (not depicted). Interestingly, *SIGLEC5* expression was increased in Sig-14$^{-/-}$ neutrophils (Fig. 3 E), likely because of promoter swapping that leads to expression of Siglec-5 under the Siglec-14 promoter (Fig. 2 A).

TLR priming increases GBS suppression of Sig-14$^{-/-}$ but not Sig-14$^{+/+}$ neutrophil responses

Because TLR-4 activation by LPS reduced the expression of Siglec-5 in Sig-14$^{+/+}$ neutrophils but increased expression in

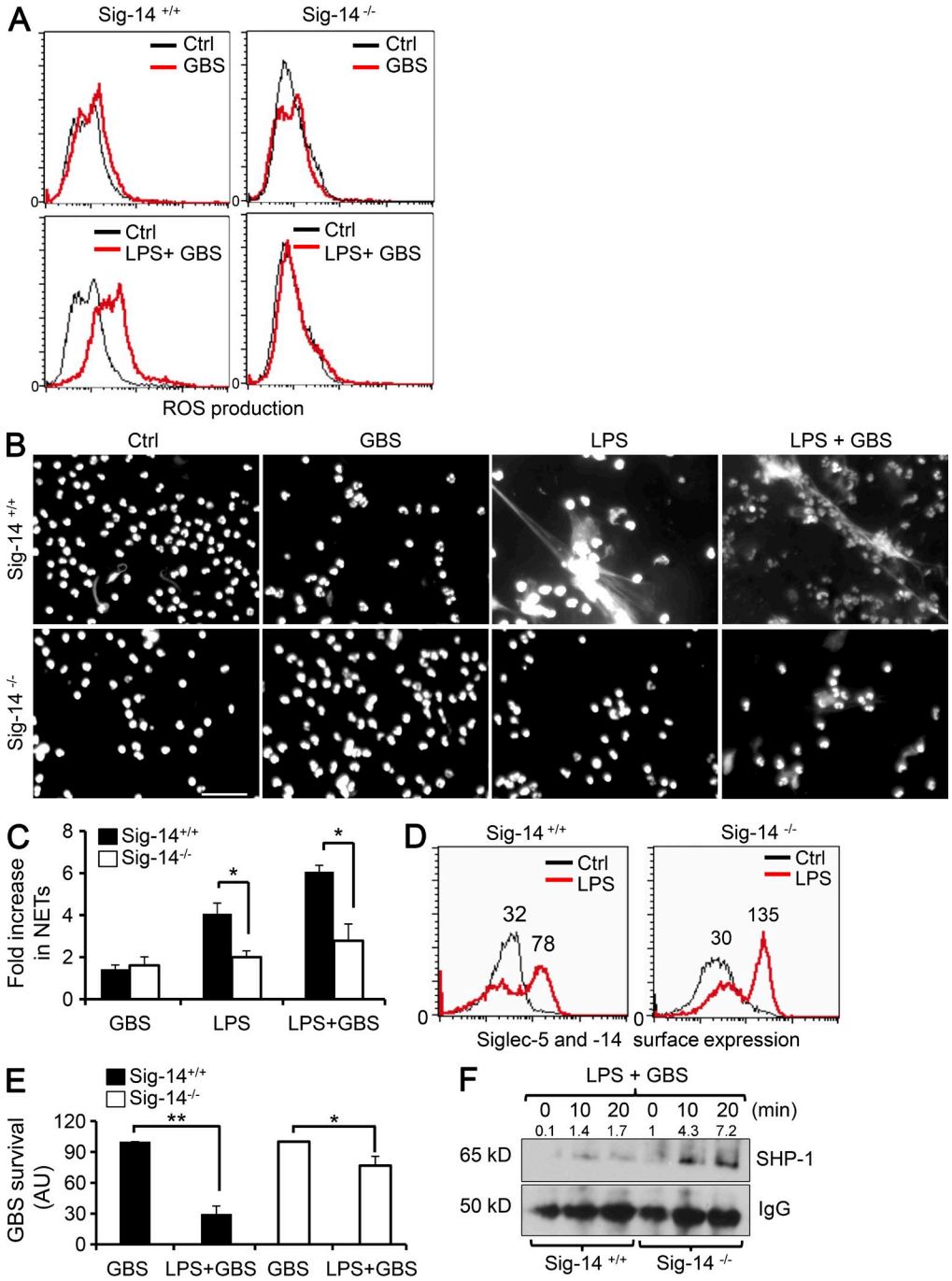


Figure 4. TLR priming increases GBS suppression of Sig-14$^{-/-}$ but not Sig-14$^{+/+}$ neutrophil responses. (A) Human neutrophils of the indicated genotypes were left unstimulated or stimulated with LPS for 6 h, incubated with H2DFCA for 30 min, and then infected with GBS (MOI = 10); ROS production was measured 20 min after infection by FACS. The histogram shows fluorescence of ROS indicator H2DCFDA. (B) Human neutrophils of the indicated genotypes were left unstimulated or stimulated with LPS for 6 h and infected with GBS (MOI = 10), and NET formation was visualized by myeloperoxidase and DAPI staining 30 min after infection. Bar, 50 µm. (C) Quantification of NETs shown in B. (D) Human neutrophils of the indicated genotypes were left unstimulated or stimulated with LPS for 8 h; expression of Siglec-5 and Siglec-14 was analyzed by FACS using antibody recognizing both human Siglec-5 and Siglec-14. The histogram depicts combined surface expression of Siglec-5 and Siglec-14. (E) Human neutrophils of the indicated genotypes were stimulated or not with LPS for 6 h and infected with GBS (MOI = 10), and bacterial killing was assayed 20 min after infection. (F) Human neutrophils of the indicated genotypes were stimulated with LPS for 6 h and infected with GBS (MOI = 10), and cell lysates prepared at the indicated times were immunoprecipitated with Siglec-5– and Siglec-14–recognizing antibody. SHP-1 recruitment and total IgG were analyzed by immunoblot. The SHP-1/IgG (×10) densitometry value is shown on the immunoblot. Data in this figure are representative of two to four independent experiments with one to three different donors per genotype in each experiment. Results are means ± SD; *, $P < 0.05$, **, $P < 0.01$.

Sig-14$^{-/-}$ neutrophils (Fig. 3 E), we hypothesized that preactivation of TLR-4 signaling in Sig-14$^{-/-}$ neutrophils would significantly increase their susceptibility to GBS-mediated immune suppression. Two phenotypes that are suppressed upon GBS Siglec-5 engagement are reactive oxygen species (ROS) generation and formation of DNA-based NET (Carlin et al., 2009a). To test our hypothesis, neutrophils of the indicated genotypes were pretreated with LPS or media control for 8 h, followed by low-dose GBS infection for 30 min. No significant increase in ROS or NET formation was observed in response to GBS stimulation alone after 30 min. However, LPS priming induced significant ROS and NET production in GBS-infected Sig-14$^{+/+}$ but not Sig-14$^{-/-}$ neutrophils (Fig. 4, A–C). Surface expression of Siglec-5 and Siglec-14 was determined by flow cytometry using an antibody that recognizes both Siglec-5 and Siglec-14 (currently no specific antibody exists that is specific for Siglec-5 only). As indicated in Fig. 4 D, an LPS-mediated increase in mean fluorescence intensity (MFI) from 32 (control) to 78 (LPS) in Sig-14$^{+/+}$ corresponds to Siglec-5/14 expression, whereas the observed increase from 30 to 135 in Sig-14$^{-/-}$ neutrophils corresponds to Siglec-5 expression alone. The increase of Siglec-5 expression in Sig-14$^{-/-}$ neutrophils may contribute, along with the absence of the activating Siglec-14 receptor, to the enhanced susceptibility of Sig-14$^{-/-}$ neutrophils to immune suppression by GBS. Consistent with this model, neutrophil killing of GBS was significantly reduced in LPS-treated Sig-14$^{-/-}$ compared with Sig-14$^{+/+}$ neutrophils (Fig. 4 E). In contrast, GBSΔβ survival was similar between the two genotypes (not depicted). To analyze the molecular events associated with Siglec receptor activation in LPS + GBS–treated neutrophils, cells were LPS pretreated or not for 8 h, followed by GBS infection for 20 min. When neutrophil lysates were immunoprecipitated by antibody recognizing human Siglec-5 and Siglec-14, followed by immunoblotting for SHP-1, we found that LPS priming + GBS infection increased recruitment of inhibitory SHP-1 in Sig-14$^{-/-}$ compared with Sig-14$^{+/+}$ neutrophils (Fig. 4 F), consistent with the initiation of inhibitory signaling through Siglec-5 (Carlin et al., 2009a).

Unexpected expression of Siglec-5 and Siglec-14 on human amniotic membranes influences inflammatory responses to GBS

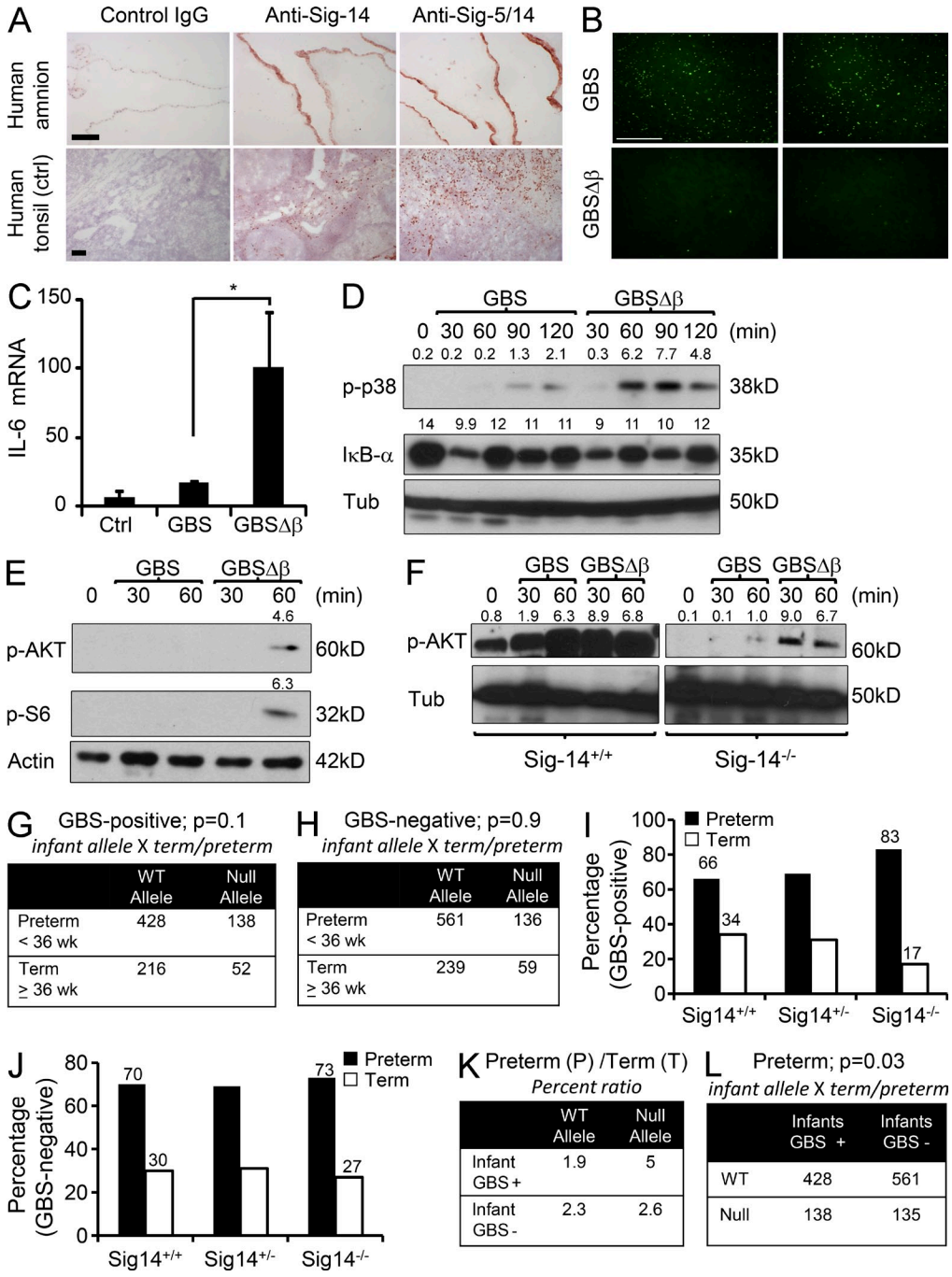
CD33-related Siglecs are mainly present on immune cells (Crocker et al., 2007). One exception is Siglec-6, which was unexpectedly found on the placental trophoblast (Brinkman-Van der Linden et al., 2007). While screening for other Siglecs in fetal tissues, we were surprised to find positive staining on the amniotic epithelial membrane (AM) of human placental sections with the antibody that recognizes Siglec-5 and Siglec-14 (Fig. 5 A). This staining is apparently unique to the human amnion, as it was not found in amnions from the closely related great apes (not depicted). Siglec-5 and Siglec-14 protein expression on human AM was confirmed by Western blot (not depicted). This unusual tissue site of Siglec human-specific expression is particularly intriguing because the human-specific pathogen GBS produces ascending infections of the placental membranes, gaining access to the amniotic fluid and fetus and leading to the potentially life-threatening early-onset pneumonia and sepsis (Edwards et al., 2011). The ability of fluorescently labeled GBS to bind to AM was established, and the key contribution of the surface-anchored Siglec-binding β-protein was confirmed (Fig. 5 B).

We next addressed the effects of the *SIGLEC14/5* polymorphism on AM responses to GBS. β-Protein–mediated binding to Sig-14$^{-/-}$ AM was accompanied by reduced IL-6 mRNA induction (Fig. 5 C). Elevated IL-6 is produced in response to cytokine stimulation and bacterial infections (Keelan et al., 1997). To determine how GBS modulates AM inflammatory responses, Sig-14$^{-/-}$ AMs were infected with GBS or GBSΔβ, lysates were prepared at the indicated times, and immunoblotting was performed for activation of signaling molecules. GBSΔβ, but not WT GBS, induced activation of p38 MAPK, AKT, and S6-mTOR pathways, as indicated by respective protein phosphorylation, indicating an important role of the β-protein in suppressing AM inflammatory responses (Fig. 5, D and E). However NF-κB activation in response to GBS, as assessed by the disappearance of IκB-α, was not affected by β-protein (Fig. 5 D).

To uncover a potential role of the *SIGLEC14/5* polymorphism in modulating GBS β-protein–mediated suppression of AM inflammatory responses, freshly collected Sig-14$^{+/+}$ and Sig-14$^{-/-}$ AMs were stimulated with either GBS or GBSΔβ, and at indicated times, the cell lysates were made and probed for AKT phosphorylation. To avoid any differences that may arise as the result of AM handling procedure in labor rooms, GBS and GBSΔβ infection–induced responses were always compared using the same AM. AKT inhibition by GBS relative to GBSΔβ was diminished in Sig-14$^{+/+}$ amnion when compared with Sig-14$^{-/-}$ amnion (Fig. 5 F). Our results suggest that Siglec-14 activation prevents the β-protein–mediated, Siglec-5–dependent immune suppression of AMs. The opposing functions of Siglec-5 and Siglec-14 represented in Fig. 5 F provide further support for a functional Siglec-5 and Siglec-14 paired receptor system.

Amniotic expression of Siglec-14 may influence the incidence of preterm birth in the context of GBS infections

Our findings reveal an important role of Siglec-14 in controlling the inflammatory response of AMs to GBS challenge. Because GBS ascending infection can precipitate premature delivery, we performed an initial genotype–phenotype correlation to probe whether the presence or absence of Siglec-14 can affect the gestation period of human infants, especially if the pregnancy was associated with a positive maternal screen for GBS rectovaginal colonization. A cohort of samples (n = 1,228; collected from Iowa [University of Iowa Hospitals and Clinics], Rochester, Pittsburgh, and Wake Forest) was divided into three groups based on available clinical information: (1) infants of GBS-positive pregnancies, (2) infants of GBS-negative pregnancies, and (3) mothers of GBS-positive pregnancies (Fig. S1 A). The three groups were analyzed for a correlation between the presence or absence of Siglec-14 and



	WT Allele	Null Allele
Preterm < 36 wk	428	138
Term ≥ 36 wk	216	52

	WT Allele	Null Allele
Preterm < 36 wk	561	136
Term ≥ 36 wk	239	59

	WT Allele	Null Allele
Infant GBS +	1.9	5
Infant GBS -	2.3	2.6

	Infants GBS +	Infants GBS -
WT	428	561
Null	138	135

Figure 5. The unusual ectopic expression of Siglec-5 and Siglec-14 on human AMs influences inflammatory responses to GBS. (A) Human AMs were stained for Siglec-5 and Siglec-14 expression using a human Siglec-5– and Siglec-14–recognizing antibody and Siglec-14 antibody by immunohistochemistry. (B) Human AMs were incubated either with FITC-labeled GBS or FITC-labeled GBSΔβ, and bacterial binding to the membrane was analyzed by fluorescence microscopy. (A and B) Bars, 50 µm. (C) Sig-14$^{-/-}$ AMs were cut into small pieces of similar size and infected either with GBS or GBSΔβ; IL-6 mRNA was analyzed by Q-RT-PCR after 2 h of infection. Results were normalized to GAPDH mRNA. Results are means ± SD; *, $P < 0.05$. (D and E) Sig-14$^{-/-}$ AMs were infected either with GBS or GBSΔβ as above. At the indicated times, cell lysates were prepared and analyzed for p38 phosphorylation and IκB-α degradation (D) and phosphorylation of AKT and S6 (E) by immunoblotting. (F) AMs of the indicated genotypes were infected either with GBS or GBSΔβ. At the indicated times, cell lysates were prepared and analyzed for phosphorylation of AKT protein by immunoblotting. The p-AKT/tubulin (×10) densitometry ratio is shown on the blot. p-AKT/tubulin, IκB-α/tubulin, and p-S6/tubulin (×10) densitometry values are shown on corresponding blots. Data in A–F are representative of two to five independent experiments with two to three different amnions per genotype in each experiment. (G–L) Human amnion genotyping: Association of the *SIGLEC14*-null allele with preterm birth in infants of *GBS*-positive pregnancies. (G and H) Tables showing distribution of WT and null alleles in term and preterm groups in infants of GBS^{+} (G) and GBS^{-} (H)

gestation period, defining preterm birth as delivery before 36 wk and term birth as 36 wk or later. Fig. 5 (G–L) and Fig. S1 show different analyses on the same cohort of samples. The genotype distribution data are shown in Fig. S1 (B–D).

Interestingly, our results showed that in GBS-positive pregnancies, preterm birth tended to be often associated with a *SIGLEC14*-null allele in infants (Fisher's exact test; infant allele × term/preterm; $P = 0.11$; Fig. 5 G). A similar correlation was not observed in the infant group with GBS-negative pregnancies (Fisher's exact test $P = 0.93$; Fig. 5 H). Although the preterm percentage in the current cohort is higher than the normal occurrence (~15% of total birth), the lack of association between the *SIGLEC14*-null allele and preterm birth in infants of GBS-negative pregnancies served as the internal negative control (Fig. 5, G–L).

In a subsequent analysis on the same cohort of samples, we examined the percentage of each genotype in term and preterm in the GBS-positive infant group. Similar to the previous result (Fig. 5 G), a higher percentage of preterm babies was observed in Sig-$14^{-/-}$ infants compared with Sig-$14^{+/+}$ infants (83 vs. 66%; Fig. 5 K). Similar correlations were not observed in the infant group with GBS-negative pregnancies or mothers of GBS-positive pregnancies (Fig. 5 J and Fig. S1 E).

We next evaluated the risk of GBS colonization in the preterm group from the same cohort, comparing Siglec genotypes. Our result demonstrates that in preterm babies ($n = 631$), GBS colonization has a statistically significant association with the *SIGLEC14*-null allele compared with the WT allele (Fisher's exact test; infant allele × GBS pos/neg infants; $P = 0.03$; Fig. 5 L). Together, our analysis of the cohort of human amnion samples indicates a regulatory role of Siglec-5 and Siglec-14 in GBS-associated amnion infections and preterm birth of human babies. Although the number of Sig-$14^{-/-}$ samples studied is lower than Sig-$14^{+/+}$ samples in all the groups (Fig. S1, B–D), no association between the *SIGLEC14*-null allele and preterm birth in infants of GBS-negative pregnancies or mothers of GBS-positive pregnancies was observed, serving as an internal negative control (Fig. 5, G–L).

DISCUSSION

Since the discovery of potentially activating CD33-related Siglecs in primates (Siglec-13, -14, -16, and 17), the question has arisen as to what their function might be. Siglec-13 and Siglec-17 were eliminated in the human lineage before the common origin of humans in Africa and possibly represent an infection-related signature of the proposed population bottleneck at that origin (Wang et al., 2012b). The two activating CD33rSiglecs that have persisted intact in humans have both undergone partial gene conversions with inhibitory counterparts (*SIGLEC14* with *SIGLEC5* and *SIGLEC16* with *SIGLEC11*; Angata et al., 2006; Crocker et al., 2007; Cao and Crocker, 2011; Wang et al., 2012a). In both instances the gene conversion events only involved the genomic segments encoding the first two domains of the proteins, thus homogenizing the ligand properties while maintaining the potential for opposing signaling functions. Based on such findings, it has been suggested (but not proven) that the 11/16 and 5/14 pairs have evolved to provide an appropriately balanced response to pathogens that seek to subvert the inhibitory versions. Here we address this hypothesis for the first time, also taking advantage of the natural human polymorphism in the Siglec-5/14 pair. In keeping with the hypothesis, our findings demonstrate that Siglec-14 counterbalances the inhibitory effects of GBS engagement to Siglec-5 on human monocytes and neutrophils. In response to LPS or GBS challenge, Siglec-14 promotes increased innate immune and inflammatory responses by activating p38 MAPK and AKT signaling pathways (while not affecting NF-κB activation).

This study also presents an unexpected finding of uniquely human Siglec-5 and Siglec-14 expression on the fetal-derived placental amnion, a tissue which would encounter GBS in the course of ascending infection of the womb during pregnancy (Doran and Nizet, 2004; Edwards et al., 2011). The inflammatory responses provoked by GBS in ex vivo challenge of AMs were likewise influenced by the presence or absence of Siglec-14. An initial genotype–phenotype correlation analysis of GBS status and gestation length of human pregnancies suggests that fetuses with the *SIGLEC14/5* polymorphism that results in Siglec-14 protein deficiency may be more likely to be born preterm if infected with GBS. The Siglec-5– and Siglec-14–dependent manifestations highlight the potential role of sialic acid in amnion–fetus biology and innate immune defense. A unique feature of our study was that most of the experiments were performed either on fresh human peripheral blood cells or amnions obtained from randomly selected donors after Siglec genotyping. Results from fresh blood cells were corroborated in a panel of cultured THP-1 cells overexpressing Siglec-5 or Siglec-14 to exclude outbreeding effects.

ITIM-bearing receptors such as SIRP-α, PIR-B, and Ly49-I are attractive targets for pathogen engagement to attenuate host immune responses (Arase and Lanier, 2004; Diacovich and Gorvel, 2010; Akkaya and Barclay, 2013). However, evolution of activating counterpart receptors like SIRP-α, PIR-A1, and Ly49-H suggests a host strategy to counterbalance pathogen manipulation (Abi-Rached and Parham, 2005; Diacovich and Gorvel, 2010; Akkaya and Barclay, 2013). Our study is the first to demonstrate in functional terms the existence of a paired receptor system in the Siglec

pregnancies. Fisher's exact test was performed using data computing infant allele × term/preterm. (I and J) Percentage of infants with various genotypes (Sig-$14^{+/+}$, Sig-$14^{+/-}$, or Sig-$14^{-/-}$) in preterm and term group; infants from GBS-positive and -negative pregnancies were analyzed in I and J, respectively. (K) Table showing the ratio of preterm to term genotype percentage obtained from I and J. (L) Association of *SIGLEC14/5* alleles with GBS colonization in preterm infants. The table shows the distribution of WT and null alleles in GBS-positive and -negative infants in the preterm group only. Fisher's exact test was performed using data computing infant allele × term/preterm.

family, where Siglec-5 and Siglec-14 invoke differing responses in peripheral blood cells as well as placental membranes upon GBS encounter. Absence of the activating paired receptor provides an advantage to the pathogen, resulting in enhanced host susceptibility to infection (Rhein et al., 2008). Accordingly, we found that Siglec-14 deficiency on neutrophils benefits microbial survival. Although an experimentally induced Siglec-5 deficiency in primary cells could not be achieved in our hands through shRNA technology, we had the opportunity to analyze Siglec-14–deficient cells from natural populations. We previously demonstrated GBS β-protein–dependent suppression of immune responses in Sig-14$^{-/-}$ neutrophils (Nordström et al., 2011). Here we found that Sig-14$^{-/-}$ AM (possessing only Siglec-5) shows less activation of immune signaling pathways upon GBS infection when compared with Sig-14$^{+/+}$ AM. Together, these findings establish that the Siglec paired receptor system plays an important role in modulating GBS pathogenesis.

Balanced activity of paired receptors is critical for effective immunity (Skokowa et al., 2005). One mechanism sustaining this balance is modulation of receptor expression, wherein the inhibitory receptors are down-regulated or modified, at which time the activating sibling may continue to operate and promote an inflammatory response (Skokowa et al., 2005; Kumar et al., 2006). The present study provides evidence that Siglec-5 and Siglec-14 mRNA expression levels are modulated in inverse directions upon LPS challenge. Although corresponding changes in receptor levels on the host cell surface cannot currently be quantified because of a lack of specific antibodies discriminating between Siglec-5 and Siglec-14, our findings do indicate that LPS-primed neutrophils, depending on Siglec-14 status, exhibit different patterns of Siglec-5 expression. We could not find in prior literature any other example of a molecule possessing this unique characteristic in which a promoter change (*SIGLEC5* under *SIGLEC14* promoter in *SIGLEC14/5* fusion) is linked to differential expression. Changes in Siglec-5 expression may contribute to differences in immune responses between Sig14$^{+/+}$ and Sig-14$^{-/-}$ neutrophils during subsequent GBS challenge.

LPS-triggered cell activation is also influenced by the surface expression of Siglec-5, -7, -9, -11, and -14 (Lock et al., 2004; Wang and Neumann, 2010; Pillai et al., 2012). There is no current evidence of direct activation of Siglecs by LPS, and therefore the mechanism for this association remains to be fully elucidated. Nevertheless, these findings help corroborate a key role for Siglec interaction with host sialic acid (e.g., interactions in -cis) in regulating the tonic activation state of leukocytes. One likely contributing factor is the ability of the Siglec-associated SHIP/DAP12 adaptor molecules to modulate downstream signaling (Wielgat et al., 2012). However, it could also be interesting to explore in future studies an additional unifying mechanism wherein Siglecs may alter the surface configuration or coaggregation of sialylated TLRs (Wielgat et al., 2012).

There are multiple potential risk factors for preterm birth, including socioeconomic status, environment, and genetic background (Doran and Nizet, 2004; Ananth and Vintzileos, 2008; Anum et al., 2009; Dolan, 2010; Tikkanen, 2011). Polymorphisms in various pro- and antiinflammatory cytokine genes are linked to differential susceptibility to prematurity (Anum et al., 2009). These factors may predispose to infection and/or provoke inflammation, events which are implicated in precipitating early labor (Anum et al., 2009). GBS is one of the leading pathogens in infection associated with preterm deliveries (Doran and Nizet, 2004), and our initial genotype–phenotype study suggests that the *SIGLEC14/5* gene polymorphism may be overrepresented in a preterm cohort among GBS-positive pregnancies. Fetuses with this polymorphism lack Siglec-14 and would be more susceptible to GBS-induced blunting of innate immune responses in leukocytes and AM. Additional functional and epidemiological studies will be needed to address this issue further.

Evolution of paired receptors may be driven by pathogens (Abi-Rached and Parham, 2005; Akkaya and Barclay, 2013) but counterbalanced by risks of inflammatory disease. The *SIGLEC14/5* polymorphism predisposes to GBS susceptibility but also reduces the risk of inflammatory COPD exacerbations in a Japanese cohort (Angata et al., 2013). Our research revitalizes the notion that coexistence of host and pathogens in association with the dynamic environment may promote evolutionary changes in the host in multiple directions.

MATERIALS AND METHODS

Bacterial strains and reagents. GBS type Ia strain A909 expressing the β-protein and its isogenic β-protein–negative mutant GBSΔβ have been described previously (Carlin et al., 2009a; Nordström et al., 2011). Fluorescent-labeled and unlabeled anti–Siglec-5 antibodies were obtained from R&D Systems (clones 194128 and 194117, respectively). Anti–Siglec-14 antibody has been characterized previously (Yamanaka et al., 2009). Immunoblot analysis was performed with antibodies recognizing phospho-p38 and phospho-AKT (Cell Signaling Technology) and IκB-α (Sigma-Aldrich).

Ethics approvals. Permission to obtain human blood and amnions was received from the Institutional Biosafety Committee of University of California, San Diego. Human volunteers provided informed consent before blood samples were obtained.

THP-1 cell activation with live GBS and LPS. The human monocytic cell line THP-1 overexpressing Siglec-5 or Siglec-14 (Yamanaka et al., 2009) was infected with various GBS variants or activated by 10 ng/ml LPS (055: B5; Sigma-Aldrich) as indicated in the figure legends. Cytokine, transcript, and protein production analysis was performed as described below.

Human neutrophil, monocyte, and amnion isolation. Neutrophils from three to six different donors per genotype were used in this study. Neutrophils were isolated from whole blood using Polymorphprep solution as previously described (Carlin et al., 2009a). Monocytes were purified from the mononuclear cell fraction of Polymorphprep by passing them through a magnetic CD14 MACS cell separation kit (Miltenyi Biotech). Freshly isolated sterile human placentas obtained from caesarian section were used for preparation of AM. AMs were carefully separated from chorion using sterile tweezers, washed with PBS three times to remove any attached red blood cells, and cut into small pieces of almost equal size (~1-cm square) for use in various assays. Neutrophils and AMs were genotyped as previously described (Yamanaka et al., 2009).

Human neutrophil, monocyte, and amnion infection with GBS. GBS was propagated in Todd-Hewitt broth (Oxoid) at 37°C without shaking

and added to neutrophils or monocytes at the indicated MOIs. Bacteria and cells were spun together at 2,000 rpm for 5 min to initiate the assay and antibiotics added 1 h after infection to suppress bacterial overgrowth. Equal sizes of AM pieces were added to the RPMI containing GBS and antibiotics added 1 h after infection.

Binding assay with pure peptides. Purified recombinant B6N (tandem) and IgA binding region (tandem) were obtained as previously described (Nordström et al., 2011). A 25-nM solution of each peptide was immobilized into the wells of a microtiter plate overnight at 4°C in 50 mM carbonate/bicarbonate buffer, pH 9.6. The wells were washed with TBST wash buffer four times and blocked with 1% BSA in TBST for 1 h at room temperature. Each well was then incubated with 1 µg/ml Siglec-Fc in TBST containing 1% BSA for 1 h at room temperature. The wells were washed four times, followed by incubation with anti–human IgG-HRP in TBST + 1% BSA. After four washes, wells were incubated in the ELISA HRP substrate 680 (LI-COR Biosciences) according to the manufacturer's instructions and then scanned by the Odyssey scanner (LI-COR Biosciences).

Binding assay with live GBS variants. 10 µg/ml recombinant Protein A was immobilized into the wells and incubated with Siglec-Fc as above. FITC-labeled GBS was prepared as above and added to the wells for 20 min at 37°C. FITC fluorescence was analyzed at Ex/Em-435/538 before and after five washes with PBS.

GBS binding to THP1 cells, neutrophils, and amnion. Log phase GBS was suspended in sodium bicarbonate buffer, pH 8.0, containing 0.1% FITC, and incubated for 1 h at 37°C. Bacteria were extensively washed in PBS to remove trace amounts of free FITC. GBS was incubated with the indicated cells on ice for 30 min, and bacterial binding was analyzed by flow cytometry using a BD FACSCalibur in the FL1 channel.

Analysis of gene expression, cytokine secretion, and cell signaling. Total cellular RNA was extracted using the RNeasy Plus kit (QIAGEN) and reverse transcribed using an iScript kit (Bio-Rad Laboratories). Real-time PCR was performed using SYBR green (Bio-Rad Laboratories) on an iQ5 machine (Bio-Rad Laboratories). All values were normalized to human GAPDH mRNA. Primer sequences are available upon request. Cytokines in culture supernatants were quantitated using ELISA kits (R&D Systems). Whole cell or amnion tissue extracts were separated by SDS-PAGE, transferred to Immobilon membranes (EMD Millipore), and analyzed by immunoblotting. Proteinase inhibitor and phosphatase inhibitors (4× each) were added to neutrophil lysates. Work with neutrophil lysates was performed at 4°C, and ∼150–200 µg of total lysate was loaded on the gel for SDS-PAGE.

Protein isolation and Western blots. GBS-infected neutrophil extracts were immunoprecipitated with anti–human Siglec-5 antibody and protein G Sepharose beads (BD). Immunoprecipitates were separated by SDS-PAGE, transferred to polyvinylidene fluoride, and probed with rabbit anti–SHP-1 antibody (Santa Cruz Biotechnology, Inc.). Immunoblots were quantified using ImageJ software (National Institutes of Health).

Immunostaining for Siglec-5 and Siglec-14 proteins on human amnion. Paraffin sections were deparaffinized, blocked for endogenous nonspecific sites, and stained with human anti–Siglec-5 and anti–Siglec-14 antibodies. Control sections were stained with IgG isotype.

NET and ROS production assay. After GBS infection, to visualize NET production, neutrophils were incubated with rabbit polyclonal antibodies against myeloperoxidase (Dako), followed by staining with DAPI and Alexa Fluor 488–conjugated goat anti–rabbit IgG (Invitrogen) as previously described (Carlin et al., 2009a). Images were recorded using an Axiovert microscope (Carl Zeiss). The total amount of neutrophils and the amount of neutrophils releasing NETs per field of view were counted in four individual images per sample. For ROS assay, cells were incubated with H2DCFDA fluorescent dye (Invitrogen) along with GBS infection; ROS production was analyzed by flow cytometry.

Statistical analysis. In ex vivo experiments, the differences in mean values between groups were analyzed using a two-tailed unpaired Student's *t* test: *, $P < 0.05$; **, $P < 0.01$. Statistical analyses on amnion samples were performed on GraphPad QuickCalcs and GraphPad Software using the two-tailed Fisher's exact test.

Online supplemental material. Fig. S1 shows the human amnion genotype–phenotype study. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20131853/DC1.

We thank Prof. Kurt Benirschke (University of California, San Diego [UCSD], La Jolla, CA) and Sandra Leon-Gracia (Clinical Research Coordinator at the Department of Reproductive Medicine, UCSD) for help in collecting fresh sterile human amnions from the UCSD Medical Center. We also thank Liwen Deng (Histology Core, UCSD) for technical assistance with amnion histology.

This work was supported by National Institutes of Health grants P01HL107150 (to A. Varki and V. Nizet) and AI057153 (to V. Nizet).

The authors declare no competing financial interests.

Submitted: 4 September 2013
Accepted: 3 April 2014

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