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Paired Siglec receptors generate opposite inflammatory responses to a human-specific pathogen

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Abstract

Paired immune receptors display near-identical extracellular ligand-binding regions but have intracellular sequences with opposing signaling functions. While inhibitory receptors dampen cellular activation by recognizing self-associated molecules, the functions of activating counterparts are less clear. Here, we studied the inhibitory receptor Siglec-11 that shows uniquely human expression in brain microglia and engages endogenous polysialic acid to suppress inflammation. We demonstrated that the human-specific pathogen *Escherichia coli* K1 uses its polysialic acid capsule as a molecular mimic to engage Siglec-11 and escape killing. In contrast, engagement of the activating counterpart Siglec-16 increases elimination of bacteria. Since mice do not have paired Siglec receptors, we generated a model by replacing the inhibitory domain of mouse Siglec-E with the activating module of Siglec-16. Siglec-E16 enhanced proinflammatory cytokine expression and bacterial killing in macrophages and boosted protection against intravenous bacterial challenge. These data elucidate uniquely human interactions of a pathogen with Siglecs and support the long-standing hypothesis that activating counterparts of paired immune receptors evolved as a response to pathogen molecular mimicry of host ligands for inhibitory receptors.

Keywords *Escherichia coli* K1; molecular mimicry; paired receptors; polysialic acid; Siglec

Subject Categories Immunology; Microbiology, Virology & Host Pathogen Interaction

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Introduction

Paired receptors are predominantly found on immune cells. They have very similar amino acid sequences within their extracellular parts, but contain elements with either activating or inhibitory properties in the transmembrane and intracellular segments (Lanier, 2001; Barrow & Trowsdale, 2006; Kuroki *et al*, 2012). The inhibitory receptors possess immunoreceptor tyrosine-based inhibitory motifs (ITIMs) within their cytoplasmic region. Phosphorylation of ITIM tyrosine residues by Src kinases generates specific binding sites for Src homology 2 (SH2) domains of phosphatases SHP-1 and SHP-2 (Tourdot *et al*, 2013). The activating counterparts do not contain ITIMs but instead associate with adaptor proteins like DAP12 with their transmembrane parts (Kameda *et al*, 2013). DAP12 contains cytosolic immunoreceptor tyrosine-activating motifs (ITAMs) that can be phosphorylated at tyrosine residues to activate MAP kinase signaling cascades, leading to production of proinflammatory cytokines (Lanier, 2009; Hirayasu & Arase, 2015).

Although many of the inhibitory receptors have been shown to recognize host ligands, targets and functions of their activating counterparts are less defined (Yamada & McVicar, 2008). Due to the similarity in their extracellular segments, paired receptors can interact with the same ligands. When this occurs, the inhibitory receptor typically binds more tightly (Lanier, 2001). This observation has suggested that the inhibitory receptors contribute to the maintenance of immunological quiescence by the recognition of self-associated molecular patterns (SAMPs) (Varki, 2011). Interestingly, many pathogens have evolved successful molecular mimicry mechanisms to bind directly to inhibitory repressors in order to suppress the immune response of the host. It has been suggested that binding of pathogens to inhibitory receptors might have driven the evolutionary selection of activating counterparts (Barclay & Hatherley,

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2008). This is consistent with the fact that many of the paired receptor families are evolving rapidly, indicative of pressure from pathogens (Vilches & Parham, 2002). However, the presumed ligands on pathogens have been difficult to identify and are currently limited to a few viral glycoproteins (Kuroki *et al*, 2012).

Paired receptors have been identified among killer cell immunoglobulin-like receptors (KIRs), leukocyte immunoglobulin-like receptors (LILRs), paired immunoglobulin-like receptors (PIRs), Fc receptors, leukocyte-associated inhibitory receptors (LAIRs), NKp46, and Siglecs (Yamada & McVicar, 2008). Siglecs (sialic acid-binding immunoglobulin-like lectins) are a subset of I-type lectins (Varki & Crocker, 2009; Macauley *et al*, 2014). The extracellular portion of each Siglec family member includes a distinct number of Ig-like domains that allows these receptors to reach sialylated ligands extending from the same cell membrane, from other cells, or bind to soluble ligands (Crocker *et al*, 2007; Linnartz *et al*, 2010; Fong *et al*, 2015). The cytosolic segment of some Siglecs contains ITIMs, and interactions with SAMPs prevent unwanted inflammatory responses under homeostatic conditions. Conversely, their disengagement releases these brakes and results in cellular activation (Chen *et al*, 2014). Other members of the Siglec family (Siglec-14, Siglec-15, and Siglec-16) do not possess ITIMs and recruit DAP12. Siglecs that recruit DAP12 are thus classified as "activating". Interestingly, the outermost extracellular segments of Siglec-5 and Siglec-14 are kept nearly identical through ongoing gene conversion events between the *SIGLEC5* and *SIGLEC14* loci, while the intracellular parts drive opposite responses (Angata *et al*, 2006). The two proteins can thus work as paired receptors in the modulation of responses to group B *Streptococci* (GBS). Bacteria inhibit phagocytosis by targeting the inhibitory Siglec-5; conversely, recognition of GBS by Siglec-14 leads to activation of MAP kinase pathway and to more efficient clearance of the pathogen (Ali *et al*, 2014). Similarly, the sialic acid-binding properties of human Siglec-11 and Siglec-16 are indistinguishable due to the nearly identical extracellular domains that are the result of gene conversion events (Cao *et al*, 2008; Wang *et al*, 2012). However, the two proteins possess intracellular domains capable of inducing opposing signals. Moreover, while the *SIGLEC11* gene is fixed in the human population, the overall *SIGLEC16* allele frequency is 0.22 and the majority of the population carry an inactive *SIGLEC16P* variant containing a four-nucleotide deletion that disrupts the open-reading frame (Cao *et al*, 2008; Wang *et al*, 2012). Lastly, previous comparative analysis has detected Siglec-11 expression in brain microglia of humans, but not in the closely related hominids, like chimpanzees (Hayakawa *et al*, 2005).

In this study, we investigated the relevance of Siglec-11 and Siglec-16 in the regulation of the innate immune response to the pathogen *Escherichia coli* K1, an important cause of meningitis in neonates and infection in the urinary tract (Wiles *et al*, 2008; Croxen & Finlay, 2010). We found it intriguing that *E. coli* K1 produces a capsular polysaccharide made of α2-8-linked sialic acids, which is a perfect mimic of the preferred ligand of Siglec-11 (Troy, 1979; Angata *et al*, 2002). It was also remarkable that *E. coli* K1 is a human-specific pathogen, and it seems to exploit a receptor (Siglec-11) that is expressed in the brain only in humans. We show here that Siglec-11 and Siglec-16 were indeed capable of modulating responses to *E. coli* K1 in opposite directions, indicating that they can act as paired receptors. To demonstrate that activating Siglecs confer better protection to bacterial infection *in vivo*, we generated a novel mouse model of human-type paired Siglec receptors by replacing Siglec-E with a chimeric receptor that includes the extracellular part of Siglec-E and the transmembrane segment of human Siglec-16. In contrast to the native ITIM-bearing inhibitory Siglec-E, the chimeric receptor Siglec-E16 was able to produce protective inflammatory responses to bacterial infection.

Results

Siglec-11 and Siglec-16 are paired receptors expressed on tissue macrophages

Siglec-11 and Siglec-16 share a high degree of amino acid sequence identity in their extracellular region, with the two outermost Ig-like domains being 99% identical (Fig 1A and B). The underlying two domains are separated from the first two domains by a short linker domain in Siglec-11. *SIGLEC11* includes an additional exon encoding a polypeptide that separates the four Ig domains from the cell membrane.

To understand the contribution of Siglec-11 and Siglec-16 to the modulation of immune responses, we studied expression of these two receptors in human tissues. First, we developed antibodies that could specifically discriminate the two proteins (Appendix Fig S1). In agreement with previous studies (Angata *et al*, 2002; Wang & Neumann, 2010; Wang *et al*, 2012), Siglec-11 was detected in spleen, lung, liver, bladder, and brain (Appendix Fig S2). Siglec-16 was expressed at generally lower levels, but detectable in spleen and the other organs, particularly in association with inflammatory states. Co-staining with CD68 indicated that these Siglecs were present on macrophages (Fig 1C). In an independent study, Siglec-11 and Siglec-16 mRNA expression was reported on human microglia, resident macrophages in the brain (Appendix Fig S3) (Sierra *et al*, 2013; Bennett *et al*, 2016). Notably, Siglec-11 and Siglec-16 could be detected on the same splenic cells (Fig 1D), indicating that inhibitory and activating receptors can be expressed in the same cells at the same time.

Pathogenic *E. coli* K1 engages Siglec-11 and Siglec-16 via its surface sialic acid capsule, generating opposite immune responses

Siglecs are found on innate immune cells that provide a first line in defense against foreign agents. To escape elimination, pathogens continuously evolve strategies to abolish recognition or rewire inflammatory responses of the host. The preferred ligands of Siglec-11 are α2-8-linked polysialic acids, which are common structures in the human brain (Angata *et al*, 2002; Wang & Neumann, 2010; Shahraz *et al*, 2015). Interestingly, *E. coli* K1, a prominent cause of bacterial meningitis in neonates, produces a capsule made of the identical α2-8-linked polysialic acids (Troy, 1979; Croxen & Finlay, 2010). The K1 capsule confers serum resistance and anti-phagocytic properties (Hoffman *et al*, 1999; Xie *et al*, 2004). Moreover, although both capsular and acapsular *E. coli* K1 strains can traverse brain microvascular endothelial cells *in vitro* and enter the central nervous system *in vivo*, only capsulated bacteria are found in positive CSF cultures and cause *E. coli* meningitis (Xie *et al*, 2004).

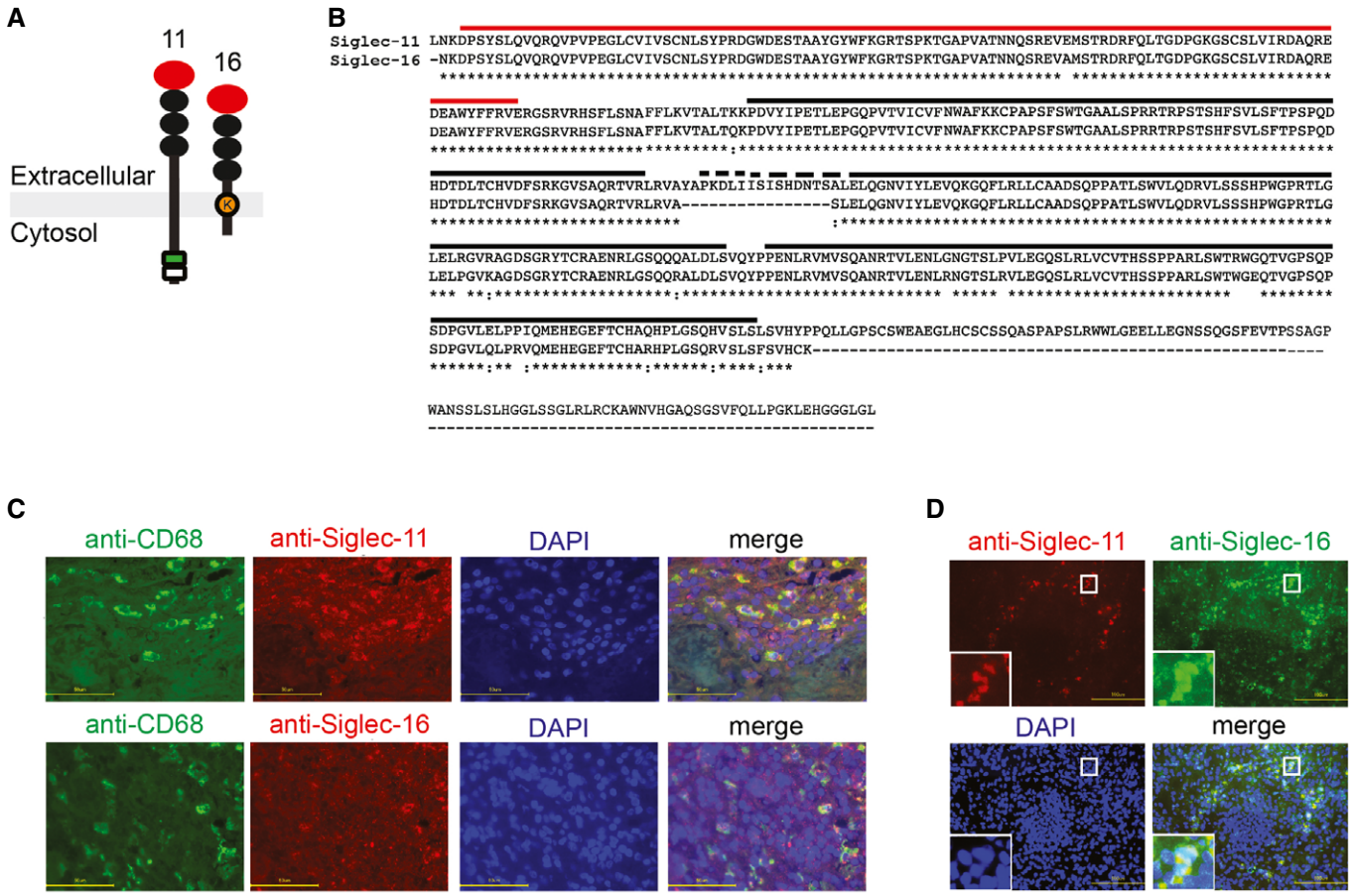

Figure 1. Siglec-11 and Siglec-16 are paired receptors expressed on macrophages in humans.

A Schematic representation of the two proteins. Filled circles represent Ig domains of the V-set (red) or CH2-type (black). Siglec-11 contains an ITIM (green box) and an ITIM-like (white box) domain in the intracellular tail. Siglec-16 has a positively charged amino acid (K, lysine) in the transmembrane span for interaction with DAP12.

B Alignment of the amino acid sequences corresponding to the extracellular parts of the two receptors. Lines indicate V-set (red) or CH2-type (black) domains.

C Co-immunofluorescence of macrophage marker CD68 and Siglec-11 or Siglec-16 in spleen samples. Cells were stained with fluorescently labeled antibodies and DAPI. The scale bar in yellow is 50 μm.

D Immunofluorescence reveals that Siglec-11 and Siglec-16 can be expressed on the same cell in spleen samples. Cells were stained with fluorescently labeled antibodies and DAPI. The insets are from higher magnification images representing the region included in the white box. The scale bar in yellow is 100 μm.

We investigated whether *E. coli* uses its capsule to hijack Siglec-11 function during bacterial infection. First, we tested whether *E. coli* K1 can engage inhibitory Siglecs. Among the protein tested, *E. coli* K1 exhibited the strongest binding to Siglec-11 (Fig 2A). A similar binding profile was observed for the activating counterpart Siglec-16, suggesting that the region responsible for bacterial binding is located within the first two near-identical extracellular domains of the two Siglecs. To understand whether the capsular sialic acid mediated Siglec binding, we compared a wild-type parent K1 strain to a mutant, deficient in sialic acid biosynthesis (Δ*neu*DB). Only the wild-type strain bound Siglec-11-Fc. Further confirmation was achieved by gain of function analysis, as introduction of the encoding biosynthesis of the K1 capsule into an unrelated non-encapsulated *E. coli* K12 stain conferred binding to the Siglec-11-Fc (Fig 2B and Appendix Fig S4). Thus, the polysialic acid capsule was necessary and sufficient for Siglec-11 binding and appeared to represent the key determinant for binding of *E. coli* K1 to Siglec-11-Fc.

To determine whether *E. coli* K1 capsule could bind Siglec-11 and Siglec-16 on a cell surface, we transfected microglial CHME-5 cells with Siglec-11 and Siglec-16 and evaluated binding of fluorescein-labeled *E. coli* K1 by flow cytometry. This analysis revealed a subpopulation of cells expressing Siglec-11 or Siglec-16 that were bound by bacteria (Fig 2C). To evaluate the contribution of Siglec-11 and Siglec-16 in innate immune response against bacteria, microglial cells were infected with *E. coli* K1 and bacterial survival was determined. Remarkably, more bacteria were recovered from cells expressing Siglec-11, while expression of Siglec-16 resulted in higher bacterial killing (Fig 2D). Siglec-11-dependent suppression of bacterial killing was not observed for the isogenic *E. coli* Δ*neu*DB mutant deficient in capsule biosynthesis. The presence of Siglec-16 reduced survival of acapsular bacteria, suggesting that additional sialic acid-independent interactions might occur between Siglec-16 and *E. coli* or that Siglec-16 altered overall cellular reactivity.

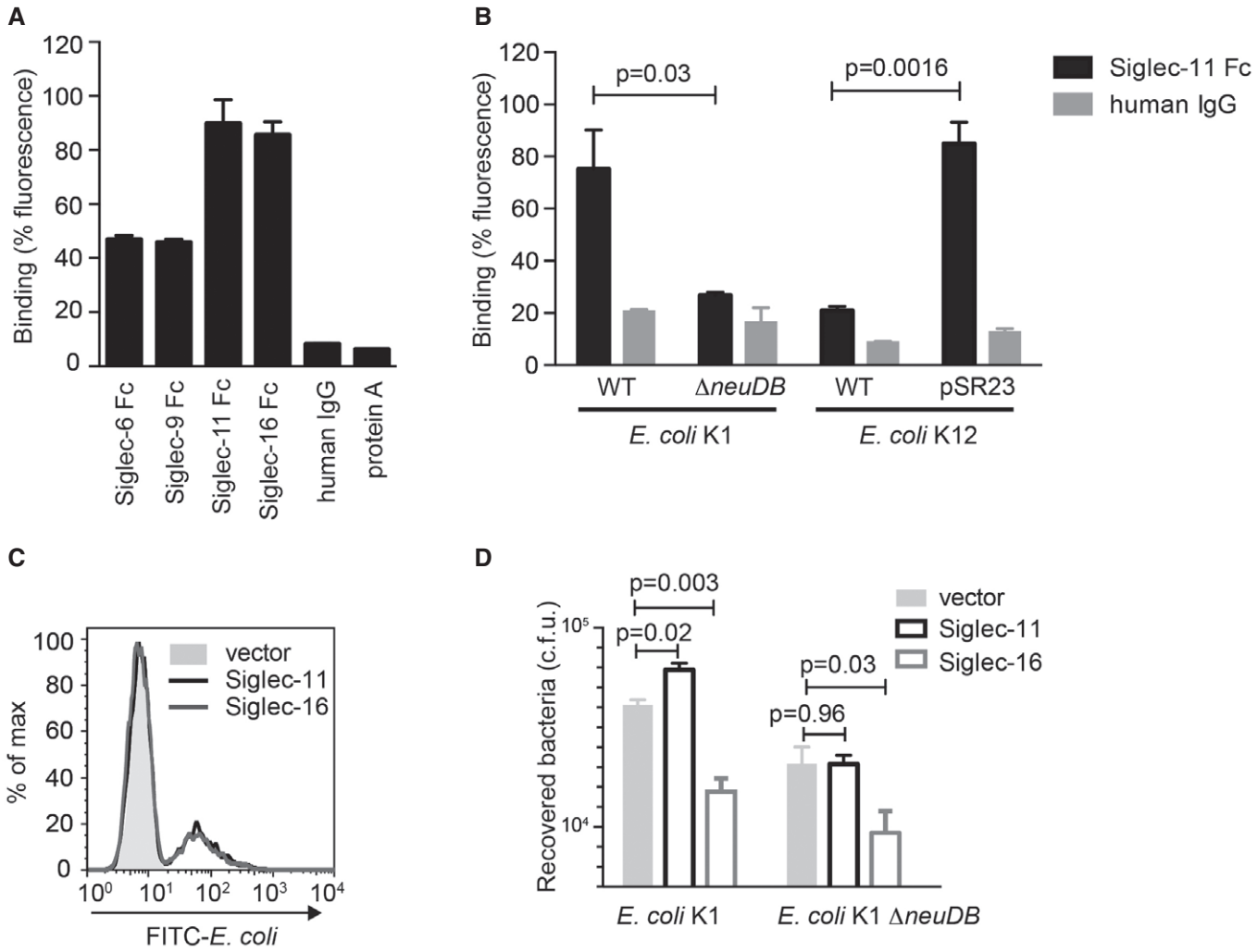

Figure 2. Human Siglec-11 and Siglec-16 bind similarly to *E. coli* K1, but drive opposite responses.

A *Escherichia coli* K1 binding to Siglec-11 and Siglec-16. Data are represented as mean ± SEM, $n = 3$.

B The polysialic acid capsule of *E. coli* K1 is necessary for binding to Siglec-11. *E. coli* K1 and an acapsular mutant (Δ*neu*DB) were tested for binding to Siglec-11. Introduction of the locus for capsule biosynthesis in *E. coli* K12 results in bacterial binding to Siglec-11. Data are represented as mean ± SEM, $n = 3$. *P*-values indicate the results of an unpaired Student's *t*-test.

C Expression of Siglec-11 or Siglec-16 on microglia CHME-5 cells results in increased bacterial adherence. The low overall extent of binding was likely due to the low efficiency of transfection of these cells.

D Bacterial killing by microglia CHME-5 cells expressing Siglecs. Recovered bacteria are indicated. Data are represented as mean ± SEM, $n = 3$. *P*-values indicate the results of an unpaired Student's *t*-test.

Engineered expression of activating Siglec receptors in mice

Studies of paired human Siglec-11/16 receptors are complicated by the weak expression on accessible primary cells, such as blood monocytes or monocyte-derived macrophages, the low population frequency of the functional *SIGLEC16* gene, and the high degree of outbreeding in the human population. Meanwhile, due to rapid evolution in the *SIGLEC* gene family in mammals, the repertoire of Siglecs differs substantially in humans and rodents, and human-type paired Siglec receptors are not found in mice. To address the relevance of paired Siglec receptors in a genetically defined *in vivo* model, we envisioned the generation of a mouse line expressing an activating receptor exhibiting ligand specificity identical to the native inhibitory murine Siglec-E (Fig 3A). First, to demonstrate that such a chimeric receptor Siglec-E16 could be expressed and signal properly, we tested its ability to recruit the DAP12. Immunoprecipitation of Siglec-E16 from lysates of cells co-transfected with Siglecs and DAP12 constructs resulted in the co-precipitation of DAP12, whereas no DAP12 was detected on immunoprecipitation of native Siglec-E (Fig 3B). We then monitored cytokine modulation by Siglec-E16. Compared to Siglec-E-expressing wild-type cells, murine macrophages with Siglec-E16 produced higher levels of proinflammatory IL-6 and lower levels of anti-inflammatory IL-10 (Fig 3C). These two experiments demonstrated that Siglec-E16 productively interacts with signaling pathways to alter inflammatory responses in cell lines.

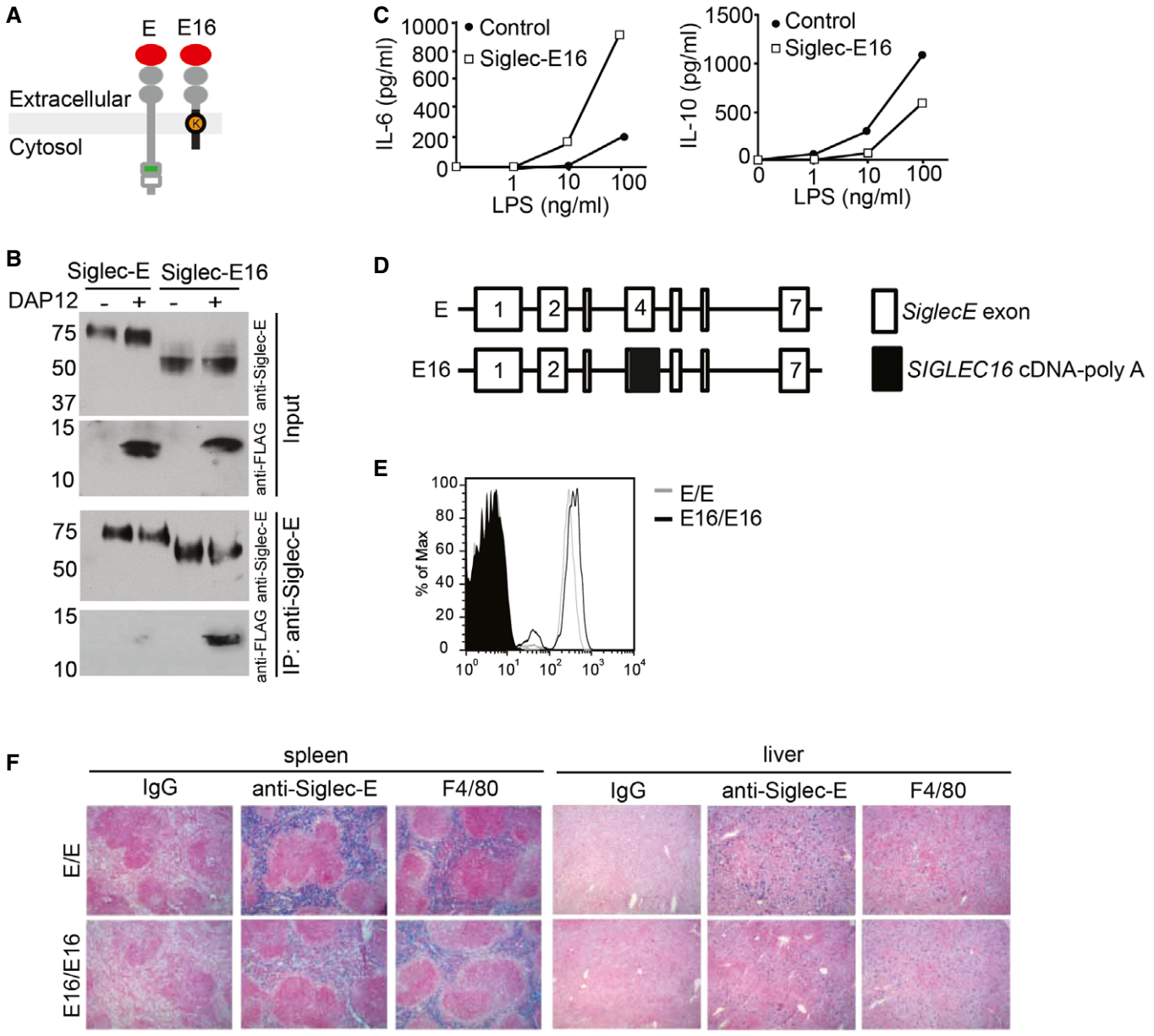

Figure 3. Engineered expression of activating Siglec-E16 in mice.

A Schematic representation of Siglec-E and Siglec-E16 receptors. The parts of Siglec-E16 derived from mouse Siglec-E or human Siglec-16 are drawn in gray and black, respectively.

B Immunoprecipitation of Siglec-E16 results in co-precipitation of DAP12. HEK293A cells were transfected with constructs for Siglecs and FLAG-tagged DAP12. Proteins were detected with anti-Siglec-E and anti-FLAG antibodies.

C Modulation of cytokine production by activating Siglec-E16. Stable RAW264.7 macrophage cell lines were stimulated with increasing doses of LPS. IL-6 and IL-10 were quantified in cell supernatants. Data are represented as mean ± SEM, $n = 3$.

D Schematic representation of the *Siglec-e* and *Siglec-e16* loci.

E Expression of Siglec-E and Siglec-E16 in blood neutrophils. Ly6G-positive cells were stained with Siglec-E (lines) or isotype control antibodies (solid).

F Expression of Siglec-E and Siglec-E16 in spleen and liver. Siglec expression is marked with blue color. Nuclei were stained with a red dye.

We then generated a mouse line E16 by insertion of a cDNA encoding for the transmembrane and cytosolic parts of Siglec-16 in exon 4 of the *Siglece* gene (Fig 3D). The insertion was designed to maintain an open reading frame between the extracellular part of Siglec-E and the transmembrane segment of Siglec-16. As transcription of Siglec-E and chimeric receptor Siglec-E16 are driven by the same promoter, we expected a similar expression pattern (Zhang *et al*, 2004). Indeed, Siglec-E16 was found on blood neutrophils at levels comparable to Siglec-E (Fig 3E). We did not detect Siglec-E or Siglec-E16 on other blood cells (data not shown). We then analyzed Siglec expression in organs and detected Siglec-E16 in spleen and liver macrophages (Fig 3F and Appendix Fig S5). Minor variations

in expression were expected, as the two receptors have differences in their transmembrane domain. Siglec-E replacement did not alter basal values of hematology and serum chemistry (Appendix Tables S1 and S2). Together, these data indicated that the chimeric Siglec-E16 exhibits an expression pattern similar to the endogenous, inhibitory Siglec-E, but drives cellular activation.

Activating Siglec receptors protect against bacterial infection

To evaluate the role of paired Siglec receptors in bacterial infection, we first tested whether *E. coli* K1 could recognize the extracellular domain that is common in Siglec-E and Siglec-E16. Siglec-E is a relatively promiscuous sialic acid-binding lectin, recognizing multiple glycans terminating in α2-3, α2-6, and α2-8 linkages. Siglec-E was shown to bind well to α2-8-disialyl oligosaccharides by glycan array (Redelinghuys *et al*, 2011). Fluorescein-labeled bacteria bound to Siglec-E to a similar extent as Siglec-11 (Fig 4A). We then studied bacterial survival in blood from homozygous wild-type (E/E) or homozygous E16 (E16/E16) mice and found enhanced killing of *E. coli* K1 in blood from homozygous E16/E16 (Fig 4B). This effect was not present using *E. coli* deficient in polysialic acid biosynthesis, suggesting that the effect was dependent on capsule interaction with the Siglecs. Furthermore, activation of MAP kinases, as measured by phosphorylation of p44/Erk1 and p38, was increased in bone marrow-derived macrophages of E16/E16 mice compared to E/E controls upon *E. coli* K1 challenge (Fig 4C). Finally, we asked whether activating Siglec-E16 conferred an advantage to the host during bacterial infection. We used an *in vivo* model of experimental hematogenous *E. coli* K1 meningitis, which mimics the pathogenesis of *E. coli* meningitis in humans and was used to study the role of the K1 capsule (Kim *et al*, 1992; Huang *et al*, 1995). In this model, bacteria are injected intravenously, resulting in bacteremia and subsequent entry of bacteria into the central nervous system. Compared to wild-type E/E mice, we observed reduced bacterial counts in blood, spleen, and liver of the E16/E16 animals (Fig 4D). We found no differences in the number of bacteria in organs with low Siglec expression, such as brain and kidneys (Appendix Fig S6). Further corroborating the immunoregulatory role of Siglec-E16 as an activating receptor, we detected higher levels of the proinflammatory cytokines IL-6, MCP-1, and IL-12 in the serum of E16/E16 animals (Fig 4E). Interestingly, a single *SigleceE16* allele could alter marginally *E. coli* K1 recovery in spleen (Appendix Fig S7). In summary, these data demonstrate that activating Siglec receptors confer an advantage to the host during infection with a bacterial species that can interact with related inhibitory Siglecs to dampen the immune responses of the host. Activating Siglec-E16 engages *E. coli* K1, leading to increased intracellular signaling and proinflammatory cytokine responses, which elicits a protective innate immune response against the pathogen.

Discussion

The innate immune system relies on receptors that distinguish molecules of the host from those of pathogens (Janeway & Medzhitov, 2002). Macrophages, neutrophils, and dendritic cells are strategically located in distinct anatomical compartments to sense conserved features of microbial pathogens via pattern-recognition proteins (Kawai & Akira, 2010; Iwasaki & Medzhitov, 2015) and mount adequate innate immune defense functions. At the same time, systems are in place to balance cellular reactivity to provide maximal protection from infection with minimal immunopathology. The molecular features and signaling properties of the inhibitory Siglecs suggest important role in balancing inflammatory responses in resting cells via recognition of host sialic acids as SAMPs (Varki, 2011; Linnartz-Gerlach *et al*, 2014; Macauley *et al*, 2014). For instance, Siglecs in microglia alleviate neurotoxicity (Wang & Neumann, 2010; Claude *et al*, 2013); CD33/Siglec-3 controls secretion of proinflammatory cytokines (Lajaunias *et al*, 2005). Engagement of such inhibitory Siglecs results in tyrosine phosphorylation within the cytoplasmic ITIM domain and recruitment of downstream phosphatases. Dephosphorylating signaling intermediates causes them to act on their respective targets to dampen inflammatory signals relayed by activating receptors (Crocker *et al*, 2007).

In this study, we investigated the role of the Siglec-11 and Siglec-16 in bacterial infection and developed a novel mouse model to demonstrate the relevance of such putative paired Siglec receptors *in vivo*. Given that the extracellular domains of Siglec-11 and Siglec-16 are very similar, we suspected they could bind similar ligands. However, whereas Siglec-11 is an inhibitory receptor, Siglec-16 was shown to associate with DAP12, suggesting that it activates inflammatory responses (Angata *et al*, 2002; Cao *et al*, 2008). It is also interesting that the two *SIGLEC* genes underwent a very unusual sequence of gene conversion events during human evolution and that *SIGLEC16* is often inactivated in the human population (Wang *et al*, 2012). We speculated that *SIGLEC16* first emerged as a countermeasure to pathogens that exploit interaction with Siglec-11 to avoid immune responses by the host. We also suspected that activating Siglecs have the long-term potential to be deleterious, perhaps by altering the inflammatory set point of cells, as their genes are frequently inactivated (Angata & Varki, 2014).

We studied Siglec-11 and Siglec-16 function in relation to a pathogen that causes meningitis and produces a capsular homopolymer made of α2,8-linked sialic acid—the same glycan structure identified as a potential ligand of Siglec-11 (Hayakawa *et al*, 2005). In this regard, it is intriguing that Siglec-11 was found in the brain of humans, but not of chimpanzee (Hayakawa *et al*, 2005) and that *E. coli* K1 is a human-specific pathogen. We demonstrated that Siglec-11 and Siglec-16 are expressed on macrophages throughout the human body, at times simultaneously on the same cell type, indicating that could behave as paired receptors. We also showed that *E. coli* K1 uses molecular mimicry strategies to engage Siglec-11 to blunt innate immune responses responsible for bacterial killing. By contrast, cellular expression of activating Siglec-16 promotes bacterial elimination. It is interesting that we observed Siglec expression in brain and bladder, which are common sites of infection of *E. coli* K1 and other uropathogenic strains (Croxen & Finlay, 2010). Similar interaction might occur with other pathogens such as *Neisseria meningitis* serotype B that produces the same capsular saccharide (Troy, 1979; Freiberger *et al*, 2007).

We then studied the relevance of human-type paired receptors in the response to bacterial infection using mice expressing engineered Siglec-E16 receptors. While the binding properties of Siglec-E16 are virtually undistinguishable from Siglec-E, it can engage DAP12 and drive proinflammatory responses, due to increased activation of MAP kinase signaling cascade. Mice expressing activating Siglec-E16 produced higher levels of proinflammatory cytokine upon

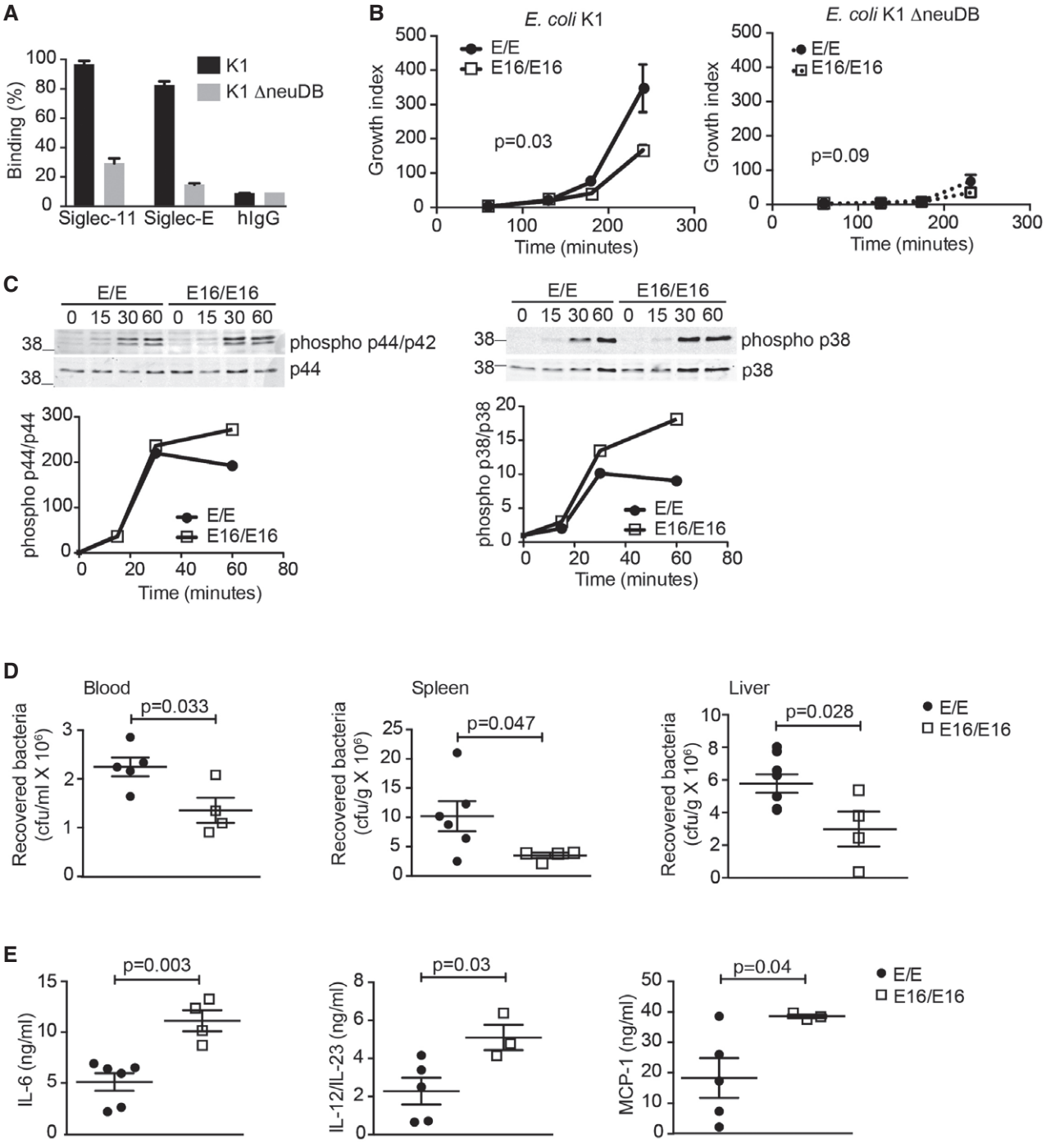

Figure 4. Activating Siglecs confer protection against *E. coli* K1 challenge.

A *E. coli* K1 binding to the extracellular part of Siglec-E. Data are represented as mean ± SEM, $n = 3$.

B *E. coli* survival in blood from E/E or E16/E16 mice, 1 h after infection. Data are represented as mean ± SEM, $n = 4$. *P*-values indicate an *F*-test between the two genotypes.

C Activation of MAP kinase signaling cascade in bone marrow-derived macrophages.

D Recovered bacteria from blood, spleen, and liver. Mean ± SEM are indicated, $n = 4$–6. *P*-values indicate the results of an unpaired Student's *t*-test.

E Cytokine levels in serum 1 h after bacterial challenge. Mean ± SEM are indicated, $n = 4$–6. *P*-values indicate the results of an unpaired Student's *t*-test.

intravenous administration of *E. coli* K1. Within an hour, animals expressing Siglec-E16 restricted bacterial dissemination, whereas *E. coli* K1 survived better when allowed to interact with the endogenous inhibitory Siglec-E. As the Siglec-E16 homozygous mice used in this study do not express inhibitory Siglec-E, future studies of animals with both activating Siglec-E16 and inhibitory Siglec-E

pairs will be useful to describe immune responses in humans with functional *SIGLEC16* or *SIGLEC14* alleles.

Building on this and previous research, we suggest that some bacterial pathogens exploit molecular mimicry of sialylated SAMPs to bind to inhibitory Siglec receptors and escape immune responses of the host. Likely to counteract such pathogen subversion, the host has evolved receptors that combine the binding properties of the inhibitory receptors to intracellular elements that activate immune responses. Similar patterns of receptor evolution have been described for activating receptors of natural killer cells (Vilches & Parham, 2002; Abi-Rached & Parham, 2005; Akkaya & Barclay, 2013). The advantage of activating receptors in protecting against pathogens would be balanced by a greater risk for unwanted inflammation, which could select against them and drive a high frequency of the non-functional alleles.

Materials and Methods

Bacteria and cell lines

Escherichia coli K1 used in this study is a spontaneous streptomycin-resistant mutant of *E. coli* RS218 (O18:K1:H7) that was isolated from the cerebrospinal fluid of a neonate with *E. coli* meningitis (Silver *et al*, 1980). *E. coli* Δ*neu*DB strain SE1634 is a *neu*DB-deficient strain that lack genes necessary for production of cytoplasmic precursors to the K1 exopolysaccharide capsule (Kim *et al*, 2003). *E. coli* K12 strain DH5α with or without the plasmid pSR23 encoding for K1 capsular polysaccharide (Silver *et al*, 1981) was also used in this study. *E. coli* were propagated in brain–heart infusion broth, BHI (Difco, BD Diagnostics, Franklin Lakes, NJ, USA) at 37°C with shaking. For all binding and infection studies, bacteria were cultivated to an optical density at 600 nm equivalent to 0.6. HEK293 and RAW264.7 were obtained from ATCC. The CHME-5 immortalized fetal microglial cell line was described in Janabi *et al* (1995).

Siglec-Fc/bacteria binding assay

Ninety-six-well plates were coated with 1 μg/well protein A (Thermo Scientific, Waltham, MA, USA) in 50 mM carbonate buffer pH 9.5 overnight at 4°C. Wells were washed with PBS-T (0.05% Tween-20 in PBS) and blocked with 1% BSA in PBS for 1 h at room temperature. 2.5 μg/well Siglec-Fcs, produced as previously described (Padler-Karavani *et al*, 2014), was incubated for 2 h at room temperature. Afterward, wells were washed with PBS-T. *E. coli* were pelleted, washed with PBS, and then incubated with 0.1% fluorescein isothiocyanate (FITC, Sigma) in PBS for 1 h at 37°C with rotation. Bacteria were extensively washed with PBS to remove trace amounts of free FITC and then resuspended in PBS at an optical density of 1. A volume of 0.1 ml of FITC-labeled bacteria was added to each well. Plates were centrifuged at 500 *g* for 10 min and incubated for 1 h at room temperature. After washing to remove unbound bacteria, the residual fluorescent intensity was measured using a SpectraMax M3 (Molecular Devices, Sunnyvale, CA, USA).

Adherence to microglia cells and killing assay

CHME-5 cells were transfected with constructs for expression of Siglecs using Neon (Life Technologies, Grand Island, NY, USA). Cells were infected with *E. coli* K1 strains at a multiplicity of infection of 0.1 bacteria per cell, followed by incubation for 30 min at 37°C. Cells were washed and lysed with 0.01% Triton X-100, and bacteria were counted by serial dilutions. For bacteria adherence assay, FITC-labeled bacteria were incubated with transfected cells. Cells were washed and analyzed by flow cytometry using a FACSCalibur (BD Biosciences, San Jose, CA, USA).

MAP kinase activation

Murine macrophages were derived from bone marrow cells cultured with conditional media obtained from culture supernatants of L929 cells for 6 days. Cells were incubated with bacteria (10^5 c.f.u./test) for different time points, washed with PBS and lysed in lysis buffer (1% NP-40, 20 mM Tris pH 8, 150 mM NaCl). Lysates were spun at 12,000 *g*. Protein concentration of the supernatant was measured with a BCA kit (Pierce). Proteins were separated by SDS–PAGE and transferred to a nitrocellulose membrane. Membranes were incubated with anti-phospho-p38 MAPK (Thr180/Tyr182) (Cell Signaling, Danvers, MA, cat 9216), anti-p38 MAPK (BioLegend, San Diego, CA, USA, cat 620422), anti-phospho-ERK1/2 (pT202/pY204) (BD Bioscience, cat 612358), or anti-p44 MAP Kinase (Cell Signaling, cat 4372).

Analysis of cytokine expression

Supernatant was collected from stable RAW264.7 cell lines expressing Siglec-E16 stimulated with LPS (Sigma) for 24 h. Serum was separated from whole blood by incubation in BD Microtainer tubes (cat 365956). IL-6 concentration was measured using an ELISA kit from R&D Systems (Minneapolis, MN). IL-10, IL-12, MCP-1, and TNF-α concentrations were measured using ELISA kits from BioLegend.

Generation of Siglec-E16 mouse line

The E16 mouse line was generated by GenOway (France) by homologous recombination of a DNA cassette including human Siglec-16 cDNA encoding for the transmembrane and intracellular tail within the exon 4 of *SIGLECE* in embryonic stem cells in C57BL/6 background.

Mouse infection model

All animal experiments were approved by the Committee on the Use and Care of Animals, UCSD, and performed using accepted veterinary standards. Ten- to twelve-week-old mice received *E. coli* RS218 (10^7 c.f.u.) in 100 μl PBS via the tail vein. One hour later, mice were sacrificed and blood was collected by cardiac puncture. Animals were then perfused with Ringer solution as described previously (Zhu *et al*, 2010). Organs were isolated from mice, homogenized with a magDNA (Roche, Basel, Switzerland), and plated for bacteria count.

Immunohistochemistry

Frozen human tissues were obtained from the National Cancer Institute funded Co-operative Human Tissue Network. Sections were blocked for endogenous peroxidases and endogenous biotin and overlaid either with control mouse IgGs (Abcam, Cambridge, MA, USA,

cat ab81032), mouse anti-CD68 (AbD Serotec, Raleigh, NC, USA, cat MCA5709), mouse anti-Siglec-11 (R&D Systems, cat MAB3258), or mouse anti-Siglec-16 (R&D Systems, clones 706004, 706022 and 706032), followed by detection using appropriate secondary reagents, and developed using Vector Blue SK4200 (Vector Laboratories, Burlingame, CA, USA), and Fast Red nuclear counterstain, following protocols of the UC San Diego Mouse phenotype Core http://mousepheno.ucsd.edu/. For immunofluorescence, mouse anti-Siglec-11 antibodies were biotinylated using Biotin–NHS (Thermo Scientific). Tissue sections were sequentially incubated with mouse anti-Siglec-16, Alexa Fuor488-conjugated anti-mouse IgG (Life Technologies), biotinylated anti-Siglec-11, and Cy3-conjugated streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Murine organs were isolated, snap-frozen in OCT, and stored at −80°C. Sections were blocked and stained with rat anti-F4/80 (AbD Serotec, cat MCA497), rat anti-Siglec-E (BioLegend, cat 677102), or rat IgG (Abcam, cat ab37361), followed by appropriate secondary reagents.

Immunoprecipitation

HEK293T expressing Siglec-E or Siglec-E16 and DAP12 were lysed, and proteins were incubated with anti-Siglec-E antibodies (BioLegend) and protein G-Dynabeads (Life Technologies). Proteins were detected with goat anti-Siglec-E antibodies (R&D Systems, cat AF5806) or rabbit anti-FLAG antibodies (Sigma). Secondary antibodies were from LI-COR (Lincoln, NE). Signals were acquired with an Odyssey instrument (LI-COR) and analyzed by Image Studio software (LI-COR).

Siglec-E16 expression on mouse cells

Blood was obtained by cardiac puncture. Bone marrow neutrophils were isolated by Percoll gradient. Splenocytes were obtained by mechanic disruption of spleen. Red blood cells were lysed by incubation in ACK buffer (Gibco). Cells were stained with anti-Siglec-E, anti-Ly6G clone 1A8 (BD Biosciences, cat 560599), or anti-F4/80 antibodies (BioLegend Inc, San Diego, cat 123115). Data were analyzed with FlowJo (FlowJo, LLC, Asland, OR, USA).

Statistical analysis

For animal experiments, mice were randomly selected and grouped, without using specific blinding procedures or exclusion conditions. The sample size (n) of each experimental group is indicated in the corresponding figure legend. Quantitative data are indicated as average ± standard error of the mean (SEM, represented as error bars). Prism 6 software (GraphPad) was used for all statistical analyses. Unpaired Student's t-test or ANOVA is indicated for comparisons involving two groups.

Expanded View for this article is available online.

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Author contributions
FS, VN, and AV wrote the manuscript. FS, IS, VN, and AV designed the experiments. FS, CSL, SS, IS, JO, and NV performed the experiments. All authors reviewed and approved this manuscript.

Conflict of interest
The authors declare that they have no conflict of interest.

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