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Siglec-E Is Up-Regulated and Phosphorylated Following Lipopolysaccharide Stimulation in Order to Limit TLR-Driven Cytokine Production1

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Abstract

Although production of cytokines by TLR is essential for viral and bacterial clearance, overproduction can be detrimental, thus controlling these responses is essential. CD33-related sialic acid binding Ig-like lectin receptors (Siglecs) have been implicated in the control of leukocyte responses. In this study, we report that murine Siglec-E is induced by TLRs in a MyD88-specific manner, is tyrosine phosphorylated following LPS stimulation, and negatively regulates TLR responses. Specifically, we demonstrate the Siglec-E expression inhibits TLR-induced NF-κB and more importantly, the induction of the antiviral cytokines IFN-β and RANTES. Siglec-E mediates its inhibitory effects on TIR domain containing adaptor inducing IFN-β (TRIF)-dependent cytokine production via recruitment of the serine/threonine phosphatase SHP2 and subsequent inhibition of TBK1 activity as evidenced by enhanced TBK1 phosphorylation in cells following knockdown of Siglec-E expression. Taken together, our results demonstrate a novel role for Siglec-E in controlling the antiviral response to TLRs and thus helping to maintain a healthy cytokine balance following infection.

On facing an immune challenge, the body's initial response involves activation of the innate immune system. One branch of this uses the pathogen recognition receptors of the TLR

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The authors have no financial conflict of interest.

Correction A correction has been published for this article. The contents of the correction have been appended to the original article in this reprint. The correction is available online at: http://www.jimmunol.org/cgi/reprint/184/3/1655

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family. In mammals, thirteen of these receptors have been identified and they recognize a diverse array of pathogen associated molecular patterns expressed by bacteria, viruses, and fungi (reviewed in Ref. 1). Once engaged, these receptors trigger a signaling cascade that ultimately leads to the activation of transcription factors such as NF-κB and members of the IFN regulatory factor family and thus the production of proinflammatory cytokines (reviewed in Ref. 2).

The production of inflammatory cytokines by these receptors is essential in controlling pathogen replication within the host. However, regulation of the response is essential to prevent pathogenesis. A number of inhibitory mechanisms have been reported to control the activation of the TLRs with the majority targeting the MyD88-dependent arm. These include IL-1 receptor associated kinase-M (3), MyD88s (4), and TRIM30α (5). Increasingly, regulators specific to TIR domain containing adaptor inducing IFN-β (TRIF)-dependent signaling have been identified, for example sterile α-and armadillo-motif containing protein (SARM)[4] (6), Ro52 (7), and TRAM adaptor with GOLD domain (8). Recently the phosphatase, PTP1B, has been identified as an inhibitor of both MyD88 and TRIF-dependent responses in macrophages (9). The importance of negative regulators of pathways that control type I IFN production is highlighted by the role these cytokines play in the pathology of the autoimmune disease systemic lupus erythematosus. Recently, TLR-7 and -9 have been demonstrated to play an important role in the overproduction of type I IFN associated with this disease. To this end, attention is very much focused on uncovering novel mechanisms for regulating TLR-driven IFN production to manipulate these responses therapeutically.

The CD33-related sialic acid binding Ig-like lectins (Siglecs) (CD33 and Siglecs 5–11), are predominantly expressed on cells of the innate immune system and have been largely shown to be inhibitory based on the presence of an ITIM in their cytoplasmic tail. Following ligation of the receptors the tyrosines within the ITIM become phosphorylated and recruit SH2-containing phosphatases such as Src homology 2 domain containing protein tyrosine phosphatase 1 (SHP1) and SHP2, thereby regulating cellular activity (10, 11). More recently, the generation of Siglec knockout mice has further strengthened their importance as inhibitory receptors. Siglec-F-deficient mice displayed enhanced eosinophilic inflammation (12) while Siglec-G-deficient mice had enhanced expansion of B1a cells (13) due to enhanced NF-κB activation (14).

To date the myeloid-specific Siglecs, human Siglec-9 and its murine orthologue Siglec-E, have been less well characterized. Recently engagement of Siglec-9 has been shown to result in reduced TNF-α production accompanied with increased IL-10 levels (15). However, involvement of these receptors in pathways leading to type I IFN production has not been demonstrated. Siglec-E is expressed mainly on cells of a myeloid lineage (16), although what regulates its expression has not yet been demonstrated. We hypothesized that, given the inhibitory nature of this family of receptors, Siglec-E expression was regulated by TLRs and

[4]Abbreviations used in this paper: SARM, sterile α-and armadillo-motif containing protein; Siglec, sialic acid binding Ig-like lectin; SHP1, Src homology 2 domain containing protein tyrosine phosphatase 1; BMDM, bone marrow-derived macrophage; GAM, goat anti-mouse; TRIF, TIR domain containing adaptor inducing IFN-β; shRNA, short hairpin RNA.

thus it had a role in modulating the immune response to TLR ligands. In this study, we show Siglec-E is induced in a MyD88-dependent manner and that once up-regulated it can control TLR-dependent NF-κB responses. Furthermore, Siglec-E recruits the negative regulator of TRIF-dependent signaling, SHP2. In keeping with Siglec-E as a negative regulator of TRIF-dependent signaling, overexpression of Siglec-E directly inhibited TLR-induced IFN-β and RANTES reporter gene activation. Notably, short hairpin RNA (shRNA) targeting Siglec-E mRNA enhanced TBK1 phosphorylation and RANTES production thus implicating Siglec-E as a novel negative regulator of TRIF dependent signaling.

Materials and Methods

Cell culture

Bone marrow-derived macrophages (BMDMs) were prepared from C57/BL mice and cultured for 1 wk in DMEM supplemented with 10% FCS, 1% pen/step, and 1% L-glutamine. Proliferation was driven by granulocyte macrophage-CSF derived from L929 supernatent. TLR3- and TLR4-HEK and 293T cells were maintained as described (7). Immortalized BMDMs from wild type, TRIF, and MyD88 knockout mice were cultured in DMEM supplemented with 10% FCS and 1% pen/strep and L-glutamine.

Plasmids and reagents

The Siglec-E plasmid and primary Ab were gifts from Prof. Paul Crocker (University of Dundee, Scotland). TLR-2, -3, and -7 ligands were supplied by InvivoGen, ultrapure *Escherichia coli* LPS by Alexis Biochemicals, and CpG from Coley Pharmaceuticals.

Immunoprecipitation and Western blotting—Lysates were generated and separated as previously described (17). They were immunoprecipitated with anti-SHP2 (Santa Cruz Biotechnology) or anti-phosphotyrosine (clone 4G10) (Upstate Biotechnology) and pY20 (Zymed). Western blots were probed with anti-Siglec-E or anti γ-tubulin (Sigma-Aldrich).

Luciferase reporter assays—Luciferase reporter assays were preformed as previously described (7).

Cross-linking Siglec-E—BMDMs were treated with 100 ng/ml LPS for 6 h before cross-linking with α-Siglec-E and goat anti-mouse (GAM) whole molecule (IgG) (Sigma-Aldrich).

RNA interference—A shRNA construct targeting Siglec-E (TCCACAGA GGAAGAGATACATTATGCGAC) or a scrambled shRNA were purchased from OriGene. RAW264.7 or BMDM cells were retrovirally infected with constructs as described previously (18). Infected cells were selected for using 4 μg/ml puromycin (Sigma-Aldrich). Siglec-E mRNA levels were determined using OneStep RT-PCR kit (Qiagen) according to the manufacturer's instructions. Following LPS stimulation, supernatants were collected and RANTES levels determined by ELISA (R&D Systems) according to the manufacturer's recommendations.

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Statistical analysis

Data shown as means ± SD of triplicates. Statistical significance was determined by one-way ANOVA with a value of $p < 0.05$ considered statistically significant.

Results

Siglec-E is induced and phosphorylated in a MyD88-dependent manner

The human orthologue of Siglec-E, Siglec-9, has been shown to act as a negative regulator of TLR signaling via the induction of the anti-inflammatory cytokine IL-10 (15). As initial observations demonstrated a low constitutive level of Siglec-E on murine BMDMs, we set out to investigate the ability of TLRs to regulate Siglec-E expression and its ability to inhibit their activity in return. A range of TLR ligands were therefore used to stimulate BMDMs and their ability to drive Siglec-E expression assessed by Western blotting. LPS stimulation of BMDMs was found to drive Siglec-E expression, with increased levels observed at 12 h and Siglec-E strongly expressed at 24 h (Fig. 1*A*). A similar pattern of induction was observed following stimulation of BMDMs with LPS derived from *Porphyromonas gingivalis* (TLR2 agonist), CpG (TLR9 agonist), and Imiquimod (TLR7 agonist) indicating that a wide range of TLR agonists can drive Siglec-E expression (Fig. 1, *B*–*D*, respectively). In contrast, stimulation of cells with the TLR3 agonist poly(I:C) was consistently unable to induce Siglec-E expression (Fig. 1*E*). Yet, we observed robust IκB degradation in response to poly(I:C) treatment (results not shown).

Given our findings, we hypothesized that Siglec-E expression may be dependent on differential usage of the adapter molecule MyD88 by the various TLRs; in particular, TLR3 does not signal via MyD88. To investigate this, immortalized BMDMs from TRIF$^{-/-}$ mice or MyD88 $^{-/-}$ mice were stimulated with LPS for 24 h. In response to LPS challenge, TRIF$^{-/-}$ BMDMs showed robust up-regulation of Siglec-E expression similar to wild-type BMDMs (Fig. 1*F*). However, LPS was unable to drive Siglec-E expression to any significant degree in MyD88$^{-/-}$ cells, confirming that Siglec-E is up-regulated by the TLRs in a MyD88-dependent manner. To date, the signaling pathways initiated by Siglecs remain poorly understood. However, previously reported inhibitory effects of Siglecs have been attributed to their ITIMs, which recruit tyrosine phosphatases following cross-linking (19). Thus, we sought to determine whether the endogenous, TLR-induced Siglec-E was phosphorylated. BMDMs were stimulated with LPS and cell lysates were immunoprecipitated using an anti-phosphotyrosine Ab. Following immunoblotting with Siglec-E, faint phosphorylation of the receptor was detected at 12 h post-LPS stimulation, whereas by 24 h the receptor was strongly phosphorylated (Fig. 1*G*). The phosphorylation of Siglec-E mirrored the induction of receptor expression, indicating that the receptor maybe rapidly engaged. This is the first time it has been shown that TLR stimulation induces Siglec-E phosphorylation and strongly indicates the up-regulated Siglec-E plays a functionally significant role in the cell.

Siglec-E expression inhibits NF-κB activation

We therefore set out to determine whether Siglec-E had any effect on TLR-driven responses. In keeping with a role for Siglec-E as a negative regulator of NF-κB activity, transient

transfection of TLR4- and TLR3-HEK293 cells with increasing concentrations of Siglec-E resulted in a dose-dependent inhibition of both LPS (Fig. 2*A*) and poly(I:C) (Fig. 2*B*) driven NF-κB reporter gene activation.

Previous studies in our laboratory have shown (10, 11) that cross-linking Siglecs family members promotes strong activation. Thus, following induction of Siglec-E expression in BMDMs by treating cells with LPS, Siglec-E was cross-linked with an anti-Siglec-E Ab and GAM secondary Ab to determine the effect of cross-linking Siglec-E on TLR-induced cytokine production. Consistently, we observed that Siglec-E cross-linking resulted in significantly reduced production of the NF-κB-dependent cytokine TNF-α ($p < 0.001$) in response to LPS as compared with the response observed in cells treated with a secondary Ab only (Fig. 2*C*). This was also seen when IL-6 production in response to LPS was examined. Again cross-linking Siglec-E resulted in a significant reduction ($p < 0.001$) in the production of IL-6 when compared with LPS alone or secondary Ab only (Fig. 2*D*). Together our results strongly suggest that the engagement and activation of Siglec-E following cross-linking can significantly impair LPS-induced NF-κB activation and subsequent TNF-α and IL-6 production.

Siglec-E regulates TRIF-dependent signaling

Although the role of Siglec family members as negative regulators of innate immune responses is well documented, their ability to influence antiviral immunity and the production of type I IFNs has yet to be determined. IFN-β is a key gene activated by both LPS and poly(I:C) in a TRIF-dependent manner (20). Thus, the effect of Siglec-E expression on IFN-β-promoter reporter gene activity was assessed. Importantly Siglec-E inhibited TRIF-driven IFN-β (Fig. 3*A*) suggesting Siglec-E acts downstream of TLR-3 and –4 in this pathway. In keeping with this, poly(I:C) stimulation of both IFN-β and RANTES reporter gene activation was dose dependently inhibited by overexpression of Siglec-E (Fig. 3, *B* and *C*, respectively).

Our results demonstrated that Siglec-E is a novel negative regulator of type I IFN induction downstream of TRIF and suggested that the up-regulation of Siglec-E by TLRs functioned to down-regulate and limit the induction of type I IFNs poststimulation. To investigate this hypothesis, we knocked down endogenous Siglec-E expression in BMDMs cells using a commercially available shRNA against Siglec-E or a scrambled control shRNA and examined its effects on the type I IFN-dependent chemokine RANTES (21). Wild-type cells and cells expressing either scrambled or Siglec-E-specific shRNA were treated with LPS for 6 h and RANTES production examined by ELISA. Enhanced RANTES production was observed in response to LPS in cells in which Siglec-E had been depleted compared with those treated with scrambled shRNA (Fig. 3*D*). Using one-way ANOVA, this was found to be significant at $p < 0.001$. Immunoblotting confirmed BMDMs stably transfected with shRNA specific to Siglec-E prevented its up-regulation in response to LPS stimulation while the scrambled shRNA had no effect (Fig. 3*D*, *bottom*). To determine whether Siglec-E expression impacted on upstream signal transduction, we examined TBK1 activation. Compellingly, depleting endogenous Siglec-E levels altered TBK1 phosphorylation (Fig. 3*E*). In RAW 264.7 cells expressing nonspecific shRNA LPS stimulation resulted in the

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phosphorylation of TBK1 at 60 and 90 min, indicating that TBK1 was activated in response to TLR4 stimulation. In cells which had been "primed" with a 24 h pretreatment of LPS, TBK1 phosphorylation was observed following pretreatment but reduced at 60 and 90 min following restimulation, indicating that TBK1 activity was down-regulated during the course of retreatment of cells with LPS. In contrast, depletion of endogenous Siglec-E resulted in constitutively phosphorylated TBK1 both in the absence of LPS and following LPS treatment, indicative of an inability of cells lacking Siglec-E to regulate TBK1 activity appropriately (Fig. 3*E*, *panel 3, lanes 1–3* and *4–6*, respectively). Siglec-E knockdown was confirmed by analyzing mRNA levels of the protein (Fig. 3*F*).

LPS induced Siglec-E can interact with SHP1 and SHP2

As with other CD33-related Siglecs, Siglec-E has been shown to interact with SHP1 and SHP2 in over-expression systems and using pervanadate pretreatment to maintain proteins in a tyrosine phosphorylated state (22, 23). SHP1 and SHP2 are protein-tyrosine phosphatases that regulates a variety of cellular processes (24, 25) and both have been reported to negatively regulate TLR signaling (26, 27). As Siglec-E was phosphorylated following TLR induction, we sought to examine whether it could recruit endogenous SHP1 and SHP2. Immunoprecipitation of Siglec-E from cells stimulated with LPS for 24 h revealed that up-regulated Siglec-E strongly associated with endogenous SHP1 (Fig. 4*A*) and SHP2 (Fig. 4*B*). Thus, this provides a potential mechanism for the observed inhibitory effects on the TLR-signaling pathways.

Siglec-E expression coincides with TLR-induced tolerance

As Siglec-E expression in BMDMs was induced by TLRs, we next examined whether Siglec-E was expressed in a physiologically relevant situation. The kinetics of Siglec-E up-regulation led us to postulate that it may play a role in endotoxin tolerance. Macrophages exhibit maximal tolerance to LPS restimulation at 24 h and this timing coincides with the up-regulation of Siglec-E (as shown in Fig. 1), thus we examined Siglec-E expression levels in various tolerance situations. As readouts of tolerance we examined IκB degradation and MAPK activation in the presence and absence of Siglec-E. To induce Siglec-E the BMDMs were pretreated with the stated ligand for 24 h and restimulated for the times shown. In parallel BMDMs were incubated with growth medium only and these are referred to as naive macrophages. As shown in Fig. 5*A* phosphorylation of JNK, p38, and ERK was apparent in naive macrophages (*lanes 2–6, panels 1, 3*, and *5*) but strongly suppressed in LPS-tolerant cells (*lanes 8–12, panels 1, 3*, and *5*). The deficient MAPK activation observed in the tolerized macrophages coincided with Siglec-E expression (*lanes 6–12, panel 7*).

Similarly, pretreating the cells for 24 h with LPS resulted in tolerance to Poly(I:C) stimulation (Fig. 5*B*). Stimulation with poly(I:C) caused JNK, p38, and ERK activation in naive BMDMs (*lanes 4* and *5, panel 1, 3*, and *5*) and this was abolished in macrophages pretreated with LPS (*lanes 10* and *11, panels 1, 3*, and *5*). Again Siglec-E expression was clearly up-regulated in these tolerized macrophages (*lanes 6–12, panel 7*).

Notably, pretreating the cells with poly(I:C) failed to inhibit subsequent LPS induced MAPK activation (Fig. 5*C*, *lanes 2–5*, compared with *lanes 8–11, panels 1, 3*, and *6*). In contrast, the

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phosphorylation of JNK and p38 was extended (*lanes 3–6* compared with *9–12, panels 1* and *3*). This lack of tolerance coincided with the absence of Siglec-E expression (*lanes 6–12, panel 7*). However, it is possible that the observed tolerance throughout the experiments is not solely Siglec-E dependent. This pattern was also observed when IκB degradation was examined under the same conditions (supplementary Fig. 1) clearly showing that poly(I:C) pretreatment did not induce LPS tolerance.

Discussion

This study is the first to demonstrate that Siglec-E is induced in a MyD88-dependent manner and is phosphorylated in response to TLR stimulation. Once up-regulated, Siglec-E inhibits NF-κB activation and represses the production of NF-κB dependent cytokines, TNF-α, and IL-6. However, the inhibitory effect of Siglec-E on TLR signaling is not limited to blockade of NF-κB activation. Significantly, phosphorylated Siglec-E is able to recruit the negative regulator of TBK1, SHP2, to turn off and limit TLR-induced IFN-β induction. Combined, our findings point toward a model in which Siglec-E is induced and subsequently acts in a negative feedback loop to suppress TLR-dependent cytokine and chemokine induction, as evidenced by the ability of Siglec-E to inhibit NF-κB, IFN-β, and RANTES reporter gene activity.

TLR-induced cytokine and chemokine induction is inhibited at multiple levels and by many mechanisms and induction of negative regulators of TLR signaling is a common means of regulating their activity. For example, the TRAIL receptor (28), ST2 (29), and MyD88s (4) are all induced upon stimulation of cells with LPS to feedback and prevent excessive TLR-driven cytokine production. We consistently observed that TLR2, 4, 7, and 9 up-regulated Siglec-E expression whereas poly(I:C) treatment did not. This differential up-regulation could suggest Siglec-E plays a precise role in the TLR signaling cascade, as it is not universally induced by all TLR ligands. This is reminiscent of TRAIL receptors which are differentially up-regulated by TLRs (28). Interestingly this is the not the first example of CD33-related Siglec expression levels being altered in immunopathology. Specifically, Siglec-F is up-regulated on eosinophils and $CD4^+$ T cells during allergic lung inflammation, suggesting that it has a nonredundant role in the negative regulation of atopy (12). Although induction of inhibitory receptors are only one level of control, their importance in regulating inflammatory cytokine production is evidenced by reports that $ST2^{-/-}$ and $SIGIRR^{-/-}$ mice are hyperresponsive to LPS challenge (29, 30). Furthermore, the clinical significance of these inhibitory receptors has recently been highlighted with the findings that SIGIRR prevents the development of murine lupus (31). Our data are the first demonstration of expression of a Siglec family member being regulated by TLRs in a MyD88-dependent manner and strongly supports a role for these receptors as being important in innate immune function. In keeping with this, overexpressed Siglec-E inhibits TLR-induced NF-κB activation and furthermore, in a more physiological context, Ab cross-linking, and hence activation of Siglec-E reduces the expression of TNF-α and IL-6 in response to TLR stimulation of cells. Consistent with previous reports showing that Siglec-G and Siglec-9 can negatively regulate NF-κB activation (14) and TLR-induced production of TNF-α (15), respectively.

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Our studies show, as demonstrated by previous in vitro overex-pression studies (22, 23), that TLR-induced Siglec-E can interact with endogenous SHP1 and SHP2. The effective transmission of the TLR-induced signaling cascade involves, in part, tyrosine phosphorylation of receptors and signaling adaptors. This is exemplified with findings that tyrosine phosphorylation of Mal is essential for it to signal (32) and inhibition of Bruton's tyrosine kinase results in decreased DNA binding ability of NF-κB in response to LPS treatment (33). Of equal importance is the control of these phosphorylation events, often by the recruitment of phosphatases such as SHP1 and SHP2. Both these proteins have been reported to be involved in negatively regulating the TLR cascade with SHP1 regulating NF-κB (27) and SHP2 regulating the TRIF-dependent signaling cascade (26). As Siglec-E is induced by TLR stimulation and can bind SHP1 and SHP2, we postulated Siglec-E is the mechanism by which these phosphatases get recruited into the signalsome to dampen the response. Because the endogenous signaling network is complex and involves numerous cross-talk pathways, we assume other signals dictate what SHP is recruited to Siglec-E thus dictating whether NF-κB or IFN regulatory factor activation is inhibited. Interestingly, we found knockdown of Siglec-E failed to impact on IκB degradation or TNF-α production (data not shown) even though we demonstrated Siglec-E could inhibit this pathway. Presumably due to the number of known inhibitors of the MyD88 signaling cascade (IL-1 receptor associated kinase-M, ST2, MyD88s), there is a degree of redundancy between these molecules and our results suggest that in the absence of Siglec-E this pathway can still be curtailed. Significantly, TRIF-dependent signaling was affected by the absence of Siglec-E, suggesting that the major function of Siglec-E is to regulate the expression of type 1 IFNs. This could allow the body to respond to a bacterial infection while not producing unnecessary antiviral cytokines. The identification of proteins that inhibit IFN induction is a relatively new field. Like Siglec-E, the TIR domain-containing adaptor SARM has recently been identified as a negative regulator of TRIF-dependent TLR signaling. Similar to our findings, Carty et al. (6) demonstrated that expression of SARM blocked gene induction "downstream" of TRIF and that depletion of endogenous SARM expression by interfering RNA led to enhanced TRIF-dependent cytokine and chemokine induction. However, subsequent studies using SARM deficient mice revealed no defects in the immune response (34).

Interestingly the kinetics of Siglec-E induction is in keeping with other receptors shown to be involved in the development of endotoxin tolerance, including TRAIL-R (28) and ST2 (29). Indeed, we find Siglec-E is expressed in BMDMs displaying a tolerant phenotype. This is an area of research that could be pursued with the development of Siglec-E deficient mice.

In conclusion, Siglec-E is induced following LPS stimulation and can subsequently negatively regulate the TLR cascade, thus helping to maintain a healthy cytokine balance following infection. Our findings that Siglec-E also regulates the production of type 1 IFNs downstream of TLRs indicates that this family of receptors may prove to be important therapeutic targets for the treatment of autoimmune diseases such as systemic lupus erythrematosus.

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Appendix

Corrections

Boyd, C. R., S. J. Orr, S. Spence, J. F. Burrows, J. Elliott, H. P. Carroll, K. Brennan, J. Ní Gabhann, W. A. Coulter, J. A. Johnston, and C. A. Jefferies. 2009. Siglec-E is up-regulated and phosphorylated following lipopolysaccharide stimulation in order to limit TLR-driven cytokine production. *J. Immunol.* 183: 7703-7709.

Two authors were omitted from the article. The correct author and affiliation lines are shown below.

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In addition, the fifth sentence of the Abstract should read "Siglec-E mediates its inhibitory effects on TIR domain containing adaptor inducing IFN-β (TRIF)-dependent cytokine production via recruitment of the tyrosine phosphatase SHP2 and subsequent inhibition of TBK1 activity as evidenced by enhanced TBK1 phosphorylation in cells following knockdown of Siglec-E expression."

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References

1. Liu AH. Innate microbial sensors and their relevance to allergy. J. Allergy Clin. Immunol. 2008; 122:846–858. quiz 858–860. [PubMed: 19000576]
2. Kenny EF, O'Neill LA. Signalling adaptors used by Toll-like receptors: an update. Cytokine. 2008; 43:342–349. [PubMed: 18706831]
3. Kobayashi K, Hernandez LD, Galan JE, Janeway CA Jr. Medzhitov R, Flavell RA. IRAK-M is a negative regulator of Toll-like receptor signaling. Cell. 2002; 110:191–202. [PubMed: 12150927]
4. Burns K, Janssens S, Brissoni B, Olivos N, Beyaert R, Tschopp J. Inhibition of interleukin 1 receptor/Toll-like receptor signaling through the alternatively spliced, short form of MyD88 is due to its failure to recruit IRAK-4. J. Exp. Med. 2003; 197:263–268. [PubMed: 12538665]
5. Shi M, Deng W, Bi E, Mao K, Ji Y, Lin G, Wu X, Tao Z, Li Z, Cai X, et al. TRIM30 α negatively regulates TLR-mediated NF-κB activation by targeting TAB2 and TAB3 for degradation. Nat. Immunol. 2008; 9:369–377. [PubMed: 18345001]

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6. Carty M, Goodbody R, Schroder M, Stack J, Moynagh PN, Bowie AG. The human adaptor SARM negatively regulates adaptor protein TRIF-dependent Toll-like receptor signaling. Nat. Immunol. 2006; 7:1074–1081. [PubMed: 16964262]
7. Higgs R, Ni Gabhann J, Ben Larbi N, Breen EP, Fitzgerald KA, Jefferies CA. The E_3 ubiquitin ligase Ro52 negatively regulates IFN-β production post-pathogen recognition by polyubiquitin-mediated degradation of IRF3. J. Immunol. 2008; 181:1780–1786. [PubMed: 18641315]
8. Palsson-McDermott EM, Doyle SL, McGettrick AF, Hardy M, Husebye H, Banahan K, Gong M, Golenbock D, Espevik T, O'Neill LA. TAG, a splice variant of the adaptor TRAM, negatively regulates the adaptor MyD88-independent TLR4 pathway. Nat. Immunol. 2009; 10:579–586. [PubMed: 19412184]
9. Xu H, An H, Hou J, Han C, Wang P, Yu Y, Cao X. Phosphatase PTP1B negatively regulates MyD88- and TRIF-dependent proinflammatory cytokine and type I interferon production in TLR-triggered macrophages. Mol. Immunol. 2008; 45:3545–3552. [PubMed: 18571728]
10. Orr SJ, Morgan NM, Buick RJ, Boyd CR, Elliott J, Burrows JF, Jefferies CA, Crocker PR, Johnston JA. SOCS3 targets Siglec 7 for proteasomal degradation and blocks Siglec 7-mediated responses. J. Biol. Chem. 2007; 282:3418–3422. [PubMed: 17138568]
11. Orr SJ, Morgan NM, Elliott J, Burrows JF, Scott CJ, McVicar DW, Johnston JA. CD33 responses are blocked by SOCS3 through accelerated proteasomal-mediated turnover. Blood. 2007; 109:1061–1068. [PubMed: 17008544]
12. Zhang M, Angata T, Cho JY, Miller M, Broide DH, Varki A. Defining the in vivo function of Siglec-F, a CD33-related Siglec expressed on mouse eosinophils. Blood. 2007; 109:4280–4287. [PubMed: 17272508]
13. Hoffmann A, Kerr S, Jellusova J, Zhang J, Weisel F, Wellmann U, Winkler TH, Kneitz B, Crocker PR, Nitschke L. Siglec-G is a B1 cell-inhibitory receptor that controls expansion and calcium signaling of the B1 cell population. Nat. Immunol. 2007; 8:695–704. [PubMed: 17572677]
14. Ding C, Liu Y, Wang Y, Park BK, Wang CY, Zheng P, Liu Y. Siglecg limits the size of B1a B cell lineage by down-regulating NFκB activation. PLoS ONE. 2007; 2(10):e997. [PubMed: 17912374]
15. Ando M, Tu W, Nishijima K, Iijima S. Siglec-9 enhances IL-10 production in macrophages via tyrosine-based motifs. Biochem. Biophys. Res. Commun. 2008; 369:878–883. [PubMed: 18325328]
16. Zhang JQ, Biedermann B, Nitschke L, Crocker PR. The murine inhibitory receptor mSiglec-E is expressed broadly on cells of the innate immune system whereas mSiglec-F is restricted to eosinophils. Eur. J. Immunol. 2004; 34:1175–1184. [PubMed: 15048729]
17. Elliott J, Lynch OT, Suessmuth Y, Qian P, Boyd CR, Burrows JF, Buick R, Stevenson NJ, Touzelet O, Gadina M, et al. Respiratory syncytial virus NS1 protein degrades STAT2 by using the Elongin-Cullin E_3 ligase. J. Virol. 2007; 81:3428–3436. [PubMed: 17251292]
18. Burrows JF, McGrattan MJ, Rascle A, Humbert M, Baek KH, Johnston JA. DUB-3, a cytokine-inducible deubiquitinating enzyme that blocks proliferation. J. Biol. Chem. 2004; 279:13993–14000. [PubMed: 14699124]
19. Crocker PR, Paulson JC, Varki A. Siglecs and their roles in the immune system. Nat. Rev. Immunol. 2007; 7:255–266. [PubMed: 17380156]
20. Fitzgerald KA, McWhirter SM, Faia KL, Rowe DC, Latz E, Golenbock DT, Coyle AJ, Liao SM, Maniatis T. IKKϵ and TBK1 are essential components of the IRF3 signaling pathway. Nat. Immunol. 2003; 4:491–496. [PubMed: 12692549]
21. McWhirter SM, Fitzgerald KA, Rosains J, Rowe DC, Golenbock DT, Maniatis T. IFN-regulatory factor 3-dependent gene expression is defective in Tbk1-deficient mouse embryonic fibroblasts. Proc. Natl. Acad. Sci. USA. 2004; 101:233–238. [PubMed: 14679297]
22. Ulyanova T, Shah DD, Thomas ML. Molecular cloning of MIS, a myeloid inhibitory siglec, that binds protein-tyrosine phosphatases SHP-1 and SHP-2. J. Biol. Chem. 2001; 276:14451–14458. [PubMed: 11278955]
23. Yu Z, Maoui M, Wu L, Banville D, Shen S. mSiglec-E, a novel mouse CD33-related siglec (sialic acid-binding immunoglobulin-like lectin) that recruits Src homology 2 (SH2)-domain-containing protein tyrosine phosphatases SHP-1 and SHP-2. Biochem. J. 2001; 353:483–492. [PubMed: 11171044]

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24. Salmond RJ, Alexander DR. SHP2 forecast for the immune system: fog gradually clearing. Trends Immunol. 2006; 27:154–160. [PubMed: 16458607]

25. Poole AW, Jones ML. A SHPing tale: perspectives on the regulation of SHP-1 and SHP-2 tyrosine phosphatases by the C-terminal tail. Cell Signal. 2005; 17:1323–1332. [PubMed: 16084691]

26. An H, Zhao W, Hou J, Zhang Y, Xie Y, Zheng Y, Xu H, Qian C, Zhou J, Yu Y, et al. SHP-2 phosphatase negatively regulates the TRIF adaptor protein-dependent type I interferon and proinflammatory cytokine production. Immunity. 2006; 25:919–928. [PubMed: 17157040]

27. An H, Hou J, Zhou J, Zhao W, Xu H, Zheng Y, Yu Y, Liu S, Cao X. Phosphatase SHP-1 promotes TLR- and RIG-I-activated production of type I interferon by inhibiting the kinase IRAK1. Nat. Immunol. 2008; 9:542–550. [PubMed: 18391954]

28. Diehl GE, Yue HH, Hsieh K, Kuang AA, Ho M, Morici LA, Lenz LL, Cado D, Riley LW, Winoto A. TRAIL-R as a negative regulator of innate immune cell responses. Immunity. 2004; 21:877–889. [PubMed: 15589175]

29. Brint EK, Xu D, Liu H, Dunne A, McKenzie AN, O'Neill LA, Liew FY. ST2 is an inhibitor of interleukin 1 receptor and Toll-like receptor 4 signaling and maintains endotoxin tolerance. Nat. Immunol. 2004; 5:373–379. [PubMed: 15004556]

30. Wald D, Qin J, Zhao Z, Qian Y, Naramura M, Tian L, Towne J, Sims JE, Stark GR, Li X. SIGIRR, a negative regulator of Toll-like receptor-interleukin 1 receptor signaling. Nat. Immunol. 2003; 4:920–927. [PubMed: 12925853]

31. Lech M, Kulkarni OP, Pfeiffer S, Savarese E, Krug A, Garlanda C, Mantovani A, Anders HJ. Tir8/Sigirr prevents murine lupus by suppressing the immunostimulatory effects of lupus autoantigens. J. Exp. Med. 2008; 205:1879–1888. [PubMed: 18644972]

32. Piao W, Song C, Chen H, Wahl LM, Fitzgerald KA, O'Neill LA, Medvedev AE. Tyrosine phosphorylation of MyD88 adapter-like (Mal) is critical for signal transduction and blocked in endotoxin tolerance. J. Biol. Chem. 2008; 283:3109–3119. [PubMed: 18070880]

33. Jefferies CA, Doyle S, Brunner C, Dunne A, Brint E, Wietek C, Walch E, Wirth T, O'Neill LA. Bruton's tyrosine kinase is a Toll/interleukin-1 receptor domain-binding protein that participates in nuclear factor κB activation by Toll-like receptor 4. J. Biol. Chem. 2003; 278:26258–26264. [PubMed: 12724322]

34. Kim Y, Zhou P, Qian L, Chuang JZ, Lee J, Li C, Iadecola C, Nathan C, Ding A. MyD88-5 links mitochondria, microtubules, and JNK3 in neurons and regulates neuronal survival. J. Exp. Med. 2007; 204:2063–2074. [PubMed: 17724133]

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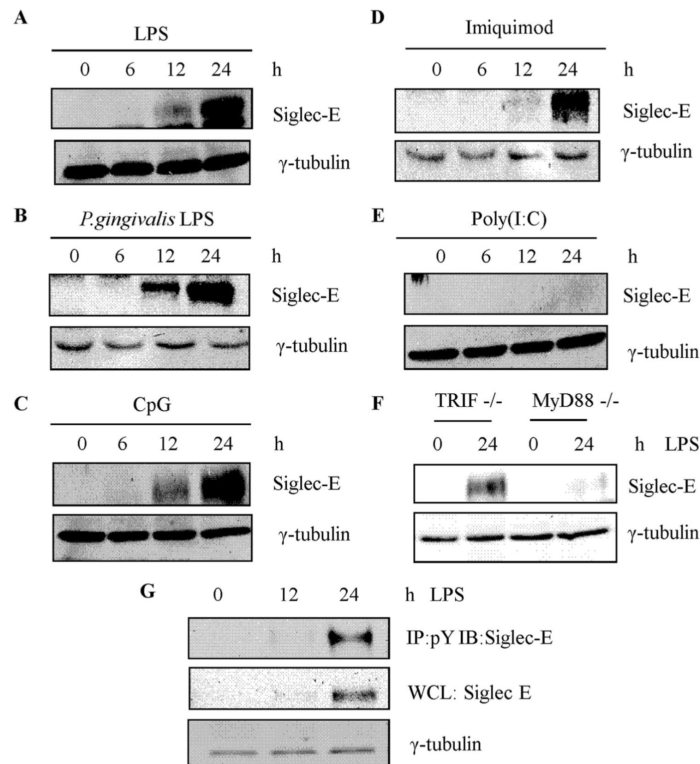

FIGURE 1.

Siglec-E is up-regulated in a MyD88-dependent manner. BMDMs were treated with 100 ng/ml LPS (*A*), 100 ng/ml *P. gingivalis* LPS (*B*), 3 μM CpG (*C*), 10 μg/ml Imiquimod (*D*), or 20 ng/ml poly(I:C) (*E*) for the indicated timepoints. Lysates were immunoblotted with Siglec-E or γ-tubulin Abs. *F*, Immortalized BMDMs derived from either TRIF$^{-/-}$ or MyD88$^{-/-}$ mice were treated with 100 ng/ml LPS for 24 h before whole cells lysate was probed for Siglec-E expression. *G*, BMDMs were treated with 100 ng/ml LPS at the indicated timepoints. Clarified lysates were then immunoprecipitated with anti-phosphotyrosine and immunoblotted for Siglec-E. Whole cell lysate was blotted for Siglec-E and γ-tubulin as a loading control. Blots are representative of three separate experiments.

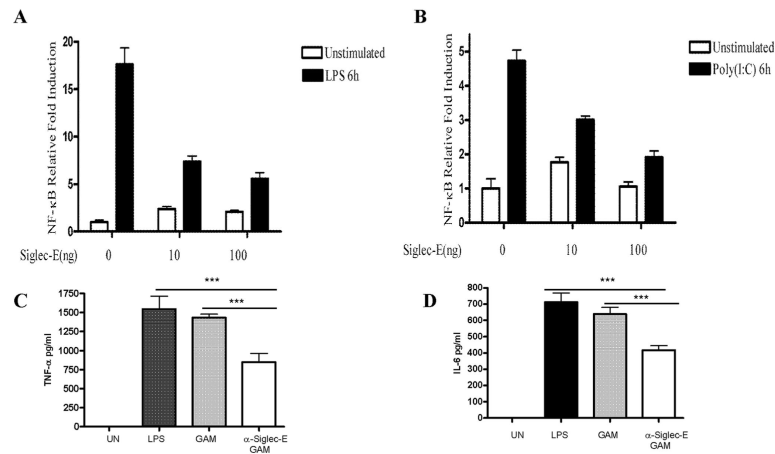

FIGURE 2.

Expression of Siglec-E inhibits NF-κB activation. *A*, TLR4-HEK cells were transfected with NF-κB reporter ± Siglec-E construct (as indicated). Eighteen hours post transfection, cells were stimulated with LPS (6 h). *B*, TLR3-HEK cells were transfected as above and stimulated with poly(I:C) for 6 h. *C*, BMDMs were stimulated with LPS to induce Siglec-E and incubated with ± α-Siglec-E and GAM with concurrent 3 h LPS stimulation. The supernatant was removed and TNF-α levels assessed by ELISA. Significance was determined by one-way ANOVA and ***, $p < 0.001$. *D*, BMDMs were treated as *C* and IL-6 levels analyzed as before. Graphs are representative of three independent experiments.

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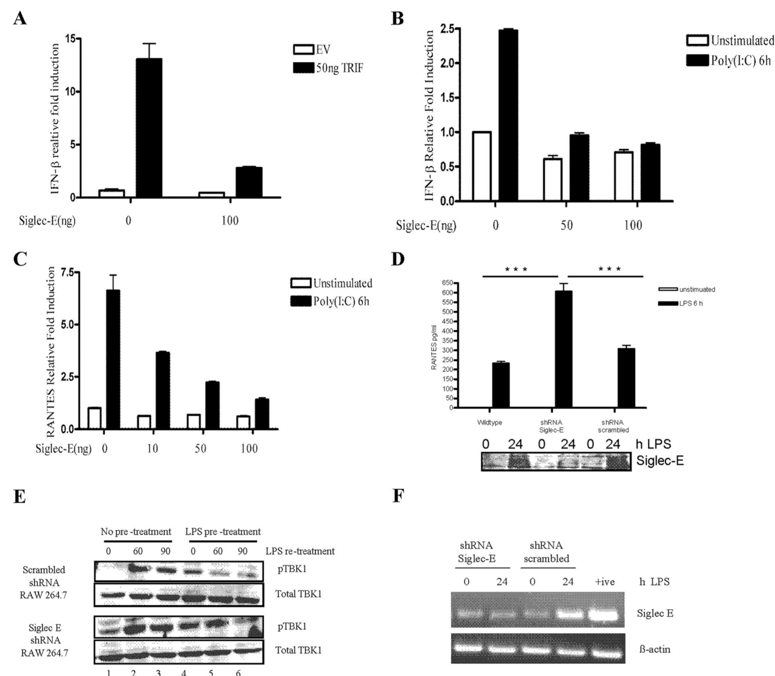

FIGURE 3.

Siglec-E regulates TRIF dependent signaling. *A*, 293T cells were transfected with IFN-*β* promoter and indicated amounts of TRIF and empty vector or Siglec-E. *B* and *C*, TLR3-HEK cells were transfected with IFN-*β* reporter (*B*) or RANTES reporter (*C*) ± Siglec-E expression construct (as indicated) and stimulated with poly(I:C) for 6 h. Luminescence (*A*–*C*) was detected using a Wallac plate reader. *D*, RANTES production was measured by ELISA from pretreated wild-type cells and cells expressing scrambled or Siglec-E specific shRNA following 6 h LPS treatment. Significance was determined by one-way ANOVA where ***, $p < 0.001$. Siglec-E depletion was confirmed by protein expression (*bottom*). *E*, RAW 264.7 cells expressing either scrambled or Siglec-E specific shRNA were stimulated with LPS as indicated. Cells lysates were immunoblotted with the indicated Abs. *F*, Siglec-E mRNA levels were detected by RT-PCR. Data representative of three independent experiments.

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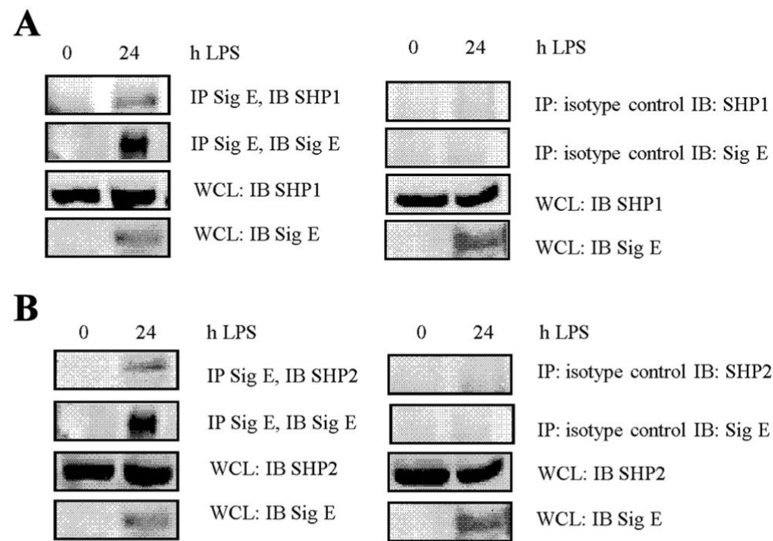

FIGURE 4.

Induced Siglec-E interacts with SHP1 and SHP2 resulting in augmented TBK1 phosphorylation. BMDMs were treated with 100 ng/ml LPS for 24 h and lysed in RIPA. Lysates were immunoprecipitated for Siglec-E and immunoblotted for SHP1 (*A*), SHP2 (*B*), and Siglec-E. The absence of any nonspecific interaction was determined by using an isotype control Ab.

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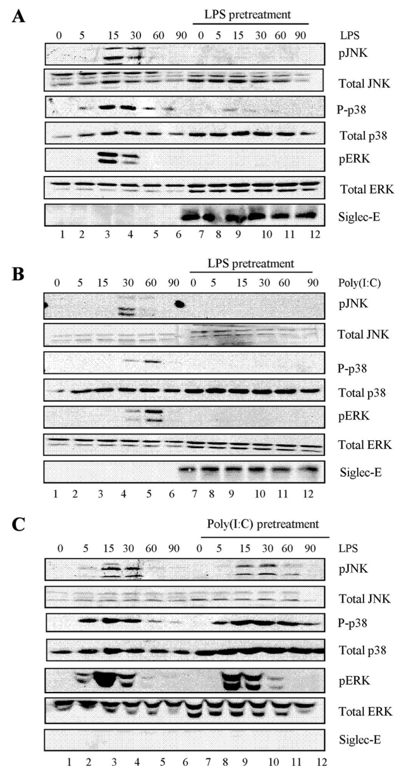

FIGURE 5.

MAPK activation is blocked by LPS pretreatment and this coincides with Siglec-E expression. BMDMs were untreated (*lanes 1–6*) or tolerized with 100 ng/ml LPS (*A* and *B*) or 25 μg poly(I:C) (*C*) for 24 h. Cells were then restimulated with the indicated ligands for the stated times and whole cell lysates were assessed for pJNK, p-p38, and pERK levels. Equal loading was confirmed by immunoblotting for total JNK, ERK, and p38 levels. The data are representative of three independent experiments.